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(57) Abstract

The present invention relates to a neuron and neural tumor growth regulatory system, based on the novel protein, arretin and its isoforms and fragments thereof, its receptor, antibodies directed against the components of this system and diagnostic, therapeutic, and research uses for each of these aspects. This protein has an apparent molecular weight of approximately 70 kDa. Embodiments of the invention comprise the amino acid sequence and probes designed therefrom for nucleic acid sequences encoding arretin. Alternatively, tagged arretin protein for use as a reporter to detect receptors of arretin, which are then sequenced and used to obtain probes for the nucleic acid sequences encoding arretin receptors, are included. The present invenetion further relates to arretin receptors and fragments thereof as well as the nucleic acid sequences coding for such arretin receptors and fragments, and their therapeutic and diagnostic uses. Substances which function as either aganists or antagonists to arretin receptors are also envisioned and included within the scope of the present invention.

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NEURON AND NEURAL TUMOR GROWTH REGULATORY SYSTEM, ANTIBODIES THERETO AND USES THEREOF

BACKGROUND

Following trauma in the adult central nervous system (CNS) of mammals, injured neurons do not regenerate their transected axons. An important barrier to regeneration is the axon growth inhibitory activity that is present in CNS myelin and that is also associated with the plasma membrane of oligodendrocytes, the cells that synthesize myelin in the CNS (see Schwab, *et al.*, Ann. Rev. Neurosci., 16, 565-595, 1993 for review). The growth inhibitory properties of CNS myelin have been demonstrated in a number of different laboratories by a wide variety of techniques, including plating neurons on myelin substrates or cryostat sections of white matter, and observations of axon contact with mature oligodendrocytes (Schwab *et al.*, 1993). Therefore, it is well documented that adult neurons cannot extend neurites over CNS myelin *in vitro*.

It has also been well documented that removing myelin *in vivo* improves the success of regenerative growth over the native terrain of the CNS. Regeneration occurs after irradiation of newborn rats, a procedure that kills oligodendrocytes and prevents the appearance of myelin proteins (Savio and Schwab, Neurobiology, 87, 4130-4133, 1990). After such a procedure in rats and combined with a corticospinal trait lesion, some corticospinal axons regrow long distances beyond the lesions. Also, in a chick model of spinal cord repair, the onset of myelination correlates with a loss of its regenerative ability of cut axons (Keirstead, *et al.*, Proc. Nat. Acad. Sci. (USA), 89, 11664-11668, 1992). The removal of myelin with anti-galactocerebroside and complement in the embryonic chick spinal cord extends the permissive period for axonal regeneration. These experiments demonstrate a good correlation between myelination and the failure of axons to regenerate in the CNS.

Until recently the identity of specific proteins important for the inhibitory activity remained elusive, although they have been sought since 1988 (Schwab *et al.*, 1993). One component of the myelin-derived inhibitors as myelin-associated glycoprotein (MAG) has been identified (McKerracher *et al.*, Neuron, 13, 229-246 and 805-811, 1994). This finding was at first surprising because MAG does not have the biochemical properties or distribution of the myelin-derived inhibitor reported by Schwab *et al.*, (1993).

There have been some expectations of the properties of the non-MAG inhibitor in myelin, based on the work of Martin Schwab (reviewed in detail by Schwab *et al.*, 1993). It was reported to be attributed to two different proteins of 35 kDa and 250 KDa. Myelin- derived growth inhibitory activity was also reported to be a property of CNS myelin but not PNS myelin. It has since been determined that PNS has inhibitory activity, but the inhibitory activity is masked by laminin (David *et al.*, 42, 594-602, 1995).

Schwab has sought to determine the identity of the myelin-derived inhibitors of neurite outgrowth, and his findings have been extensively reviewed (Schwab *et al.*, 1993). Schwab determined a possible molecular weight of the growth inhibitory proteins in the following way. Myelin proteins were separated by SDS PAGE under denaturing conditions, the gel was cut into slices and proteins were eluted from the slices and inserted into liposomes. The liposomes were tested for inhibitory activity. Regions of the gel corresponding to 250 kDa and 35 kDa were identified as most inhibitory, and heat destroyed the inhibitory activity. The loss of activity with heat suggested that the activity was due to a protein that required native conformation. Why this putative protein retains biological activity after the denaturing conditions of SDS-PAGE remain a mystery. The evidence to claim the 250 kDa and 35 kDa proteins as the major myelin inhibitors is weak.

The evidence for the 250 kDa and 35 kDa proteins as myelin-derived inhibitors comes

mainly from the work of Schwab with their IN-1 antibody. Schwab raised monoclonal antibodies to the inhibitory proteins eluted from gels and cloned one monoclonal antibody, called IN-1, which is a low-affinity IgM. It has been used to characterize the myelin-derived inhibition. The antibody is reported to bind to the 35 kDa and 250 kDa proteins, but the Western blots indicate that it lacks specificity and that many additional bands are also recognized (Caroni and Schwab, Neuron, 1, 85-96, 1988). The immunoprecipitation data presented in the same publication was given in tabular form rather than by showing the gels, as a rigorous analysis requires, and these data cannot be easily evaluated. However, application of the antibody to various *in vitro* preparations has been shown to partially block the inhibitory properties of myelin. Also, the application of this antibody *in vivo* allows a small number of corticospinal axons to elongate long distances after CNS injury (Schnell and Schwab, Nature, 343, 269-272, 1990; Schnell *et al.*, Nature, 367, 170-173, 1994). Moreover, raphe spinal serotonergic neurons also regenerate, and there is improvement in some aspects of locomotor function (Bregman *et al.*, Nature, 378, 498, 1995). Therefore, the evidence to date suggests that blocking the myelin-derived inhibitors of neurite outgrowth will be an important component of any therapeutic strategy to improve regeneration in the adult CNS. Because the proteins identified by the antibodies have not been identified, the components of myelin that block axon growth, in addition to MAG, remain unknown. It has been noted that both MAG and the new inhibitor arretin, that is described herein, appear to be acidic proteins. Therefore, to date, the identity of the non-MAG inhibitory components of myelin remain unknown, and the proteins that the IN-1 antibody recognizes remain uncharacterized.

While the findings of MAG as an inhibitor of neurite outgrowth were surprising, other laboratories have now substantiated our *in vitro* documentation that MAG is an important myelin-derived inhibitor of neurite growth (Mukhopadhay *et al.*, Neuron, 13, 757-767, 1994; Schafer *et al.*, Neuron, In press, 1996; DeBellard, Mol. Cell Neurosci., 7, 7616-7628, 1996). The contribution of MAG has also been examined *in vivo*, and the results indicate that other growth inhibitory proteins in myelin exist (Li *et al.*, J.

Neurosci. Res., In press, 1996). In these studies it has been shown that some differences occur in axon extension after lesions in MAG null mutant mice, a finding that differs from that reported for a similar study of a different line of MAG-deficient mice (Bartsch *et al.*, Eur. J. Neurosci., 7, 907-916, 1995; Bartsch *et al.*, Neuron, 15, 1375-1381, 1995). In both cases, however, the results from the studies of MAG knock out mice injured in the CNS are less dramatic than reported with treatment with the IN-1 antibody (Bartsch *et al.*, 1995 - see below), suggesting the non-MAG inhibitors that remain in CNS myelin form an important barrier to regeneration; indeed their expression in the absence of MAG expression may have been upregulated during CNS development.

Data has suggested that MAG may not be acting alone. To date, the presence of another protein had not been shown nor were its properties known. The present invention has, for the first time, demonstrated the presence and properties of such a protein.

Tenascins

Four members of the tenascin family have been identified and characterized: tenascin-C,
tenascin-R, tenascin-X and tenascin-Y (Bristow *et al.*, Cell Biol., 122, 265-278, 1993; Erickson, H.P., J. Cell Biol., 120, 1079-1081, 1993). Tenascin-X and tenascin-Y are not prominent in the nervous system. Tenascin-C is important in the development of the nervous system and it is the best characterized member of this protein family. It is generated by alternative splicing (Weller *et al.*, J. Cell Biol., 112, 355-362, 1991; Sriramarao and Bourdon, Nucl. Acids Res., 21, 347-362, 1993) and the variants are expressed both in the nervous system and in several non-neural tissues. Tenascin-C has been suggested to be involved in neuron-glia adhesive and migratory events and to promote axon outgrowth after injury of peripheral nerves.

Tenascin-R (TN-R), has a modular structure similar to TN-C, previously designated J1-160/180 and janusin in rodents, or restriction in chicken (Pesheva *et al.*, J. Cell

Biol., 109, 1765-1778, 1989; Fuss *et al.*, J. Neurosci. Res., 29, 299-307, 1991, and J. Cell Biol., 120, 1237-1249, 1993). Tenascin-R is predominantly expressed by oligodendrocytes during the onset and early phases of myelin formation and remains detectable in myelin-forming oligodendrocytes in the adult, and is also expressed by neurons (Pesheva *et al.*, 1989; Fuss *et al.*, 1993). Tenascin-R has been shown to be involved in promotion of neurite outgrowth and morphological polarization of differentiating neurons when presented as a uniform substrate (Lochter and Schachner, J. Neurosci., 13, 3986-4000, 1993; Lochter *et al.*, Eur. J. Neurosci., 6, 597-606, 1994). When offered as a sharp substrate boundary with a neurite outgrowth conducive molecule, tenascin-R is repellent for growth cone advance (Taylor *et al.*, J. Neurosci. Res., 35, 347-362, 1993; Pesheva *et al.*, 1993).

Tenacins are not thought to be an important component of the myelin-derived inhibitory activity because they lack the specific myelin distribution, they are not restricted to the CNS, and their molecular weight differs from the presumptive proteins identified by Schwab. However, studies have indicated that both tenascin R and tenascin C are minor inhibitory components of octlyglucoside extracts of myelin. The data indicate that growth inhibitory proteins from the CNS matrix may become associated with isolated myelin fragments.

Chondroitin Sulfate Proteoglycans (CSPGs)

Proteoglycans (PGs) are proteins that are found predominantly on the cell surface and in the extracellular matrix; they are covalently bound to complex carbohydrates called glycosaminoglycans. Glycosaminoglycans (GAGs) are polymers of disaccharide repeats, which are mostly highly sulphated and negatively charged. The main glycosaminoglycans in PGs are chondroitin sulfate, dermatan sulfate, heparan sulfate, and keratan sulfate. (Ruoslahti, E., Ann. Rev. Cell Biol., 4, 229-255, 1988). The number of GAG chains can vary from one to over one hundred.

Proteoglycans are known to be important for the development and regeneration of the

nervous system, but they have not been considered to be myelin proteins or form part of the growth inhibitory activity of myelin. Moreover, proteoglycans have not been reported to be recognized by the IN-1 antibody or to form a major growth inhibitory component of white matter regions of the CNS.

Chondroitin sulfate proteoglycans (CSPGs) constitute the major population of PGs in the CNS. The different patterns of localization and developmental expression of CSPGs throughout the nervous system implicate them in diverse roles in development and in regeneration. After injuries in the adult CNS, CSPGs are thought to be important in the formation of the glial scar. They have been implicated as both positive and negative modulators of axonal growth. Recent observations indicate that DSD-1-PG, a neural chondroitin sulfate proteoglycan, promotes neurite outgrowth of embryonic day 14 mesencephalic and embryonic day 18 hippocampal neurons from rat (Faissner *et al.*, J. Neurochem., 54, 1004-1015, 1994). However, NG2, an integral membrane CSPGs expressed on the surface of glial progenitor cells, inhibits neurite growth. The NG2 proteoglycan also inhibits neurite growth after digestion with chondroitinase ABC, indicating that the inhibitory activity is a property of the core protein and not the covalently attached chondroitin sulfate glycosaminoglycan chains (Dou and Levine, J. Neurosci., 14, 7616-7628, 1994), but for many other types of CSPGs the inhibitory activity resides in the glycosaminoglycan. Chondroitin sulfate proteoglycan immunoreactivity is increased after cerebral cortical (McKeon *et al.*, J. Neurosci., 11, 3398-3411, 1991), spinal (Pindzola *et al.*, Dev. Biol., 156, 34-48, 1993) and optic nerve lesions (Brittis *et al.*, Science, 255, 733-736, 1992). *In vitro* studies indicate that CSPG immunoreactivity on astrocytes increases when they are plated on monolayers of leptomeningeal cells (Ness and David, Glia, In press, 1997). Similar increases in CSPG immunoreactivity have been reported on Schwann cells co-cultured with astrocytes (Ghimikar and Eng, Glia, 14, 145-152, 1995). This highly sulfated proteoglycan which is a potent inhibitor of neurite growth in vitro (Snow *et al.*, Neurol, 109, 111-130, 1990), has been shown to be involved in the differentiation of developing retinal ganglion cells, and by acting as an inhibitory substrate serves to appropriately

guide ganglion cell axons toward the optic disc (Brittis and Silver, Proc. Nat. Acad. Sci. USA., 19, 7539-7542, 1992). McKeon *et al.,* J. Neurosci., 11, 3398-3411, 1991) have reported that astrocytes harvested from the site of cerebral cortical lesions express increased amounts of CSPG, which reduces neurite growth on these cells in vitro. The expression of CSPG on the surface of a subset of cultured astrocytes has also been shown to correlate with their reduced capacity to support neurite growth (Meiners *et al.*, J. Neurosci., 15, 8096-8108, 1995). The collapse of the growth cone is an important response of the growing exon to inhibitory cues in the environment. Collapse of the lamellipodium is sometimes followed by retraction of the neurite (Kapfhammer and Raper, J. Neurosci., 7, 201-212, 1987; Raper and Grunewald, Exp. Neurol., 109, 70-74, 1990; Bandtlow *et al.,* J. Neurosci., 10, 3837-3848, 1990). Many previously characterized inhibitory molecules found in the developing nervous system have been shown to cause growth cone collapse in vitro (Davies *et al.*, Neuron, 4, 11-20, 1990; Stahl *et al.*, Neuron, 5, 735-743, 1990; Bandtlow *et al.*, 1990; Keynes *et al.*, Ann. N.Y. Acad. Sci. 633, 562, 1991; Luo *et al.*, Cell, 75, 217-227, 1993). Such collapsing activity has been observed previously in the adult chicken brain and shown to bind to PNA, and be associated with glycoproteins with molecular weights of 48 and 55 kDa (Keynes *et al.*, 1991). Others, such as the 33 kDa inhibitor in the developing chicken tectum also binds to PNA (Stahl *et al*, 1990). Because proteoglycans are a very heterogenous class of proteins with diverse biological activities it is essential that individual, identified proteins be considered. Relevant to the present invention are the proteoglycans phosphocan and versican, because the protein of the present invention, arretin, has common immunological eptitopes with these proteins.

Phosphacan.

Phosphacan is a proteoglycan in brain recognized by the 3F8 antibody (Maurel *et al.*, Proc. Nat. Acad. Sci. USA, 91, 2512-2516, 1994), and by the 6B4 antibody (Maeda *et al.*, Neurosci., 67, 23-35, 1995). Phosphacan is a splice variant of a receptor-type protein tyrosine phosphatase, although phosphacan itself lacks the phosphatase domains. It is a protein with an apparent molecular weight of approximately 500 kDa,

having a core glycoprotein of approximately 400 kDa. The HNK-1 monoclonal antibody recognizes a 3-sulphated carbohydrate epitope, and this epitope is strongly represented in phosphacan from 7-day brain, but not in adult brain (Rauch *et al.*, J. Biol. Chem., 266, 14785-14801, 1991). In development phosphacan is immunostained on radial glia and on neurons (Maeda *et al.,* 1995) and generally it is expressed in both white matter and grey matter regions (Meyer-Puttlitz, *et al.*, J. Comp. Neurol. 366, 44-54, 1996). and therefore, unlike the myelin inhibitors, it is not localized only to white matter areas. It appears to be synthesized only by astroglia (Engel *et al*, 1996).

Versican.

Versican, a CSPG originally isolated from fibroblasts, also called PG-M, has an apparent molecular weight of approximately 900 kDa, with a core protein of approximately300 to 400 kDa (Braunewell *et al.*, Eur. J. Neurosci., 7, 792-804, 1995; Naso *et al*, 1994). Versican belongs to a family of aggregating CSPGs; other members of the family include the cartilage-derived aggrecan, and two PGs expressed in the nervous system, neurocan and brevican (Dours-Zimmermann and Zimmerermann, J. Biol. Chem., 269, 32992-32998, 1994). Versican is widely distributed in adult human tissues, associated with connective tissue of various organs, in certain muscle tissues, epithelia, and in central and peripheral nervous tissues. Four versican isoforms are known (Vo, V1, V2, V3), derived by alternative splicing. They vary in calculated mass from approximately 370 kDa (Vo) to approximately 72 kDa (V3). It has been suggested that the association of versican expression with cell migration and proliferation *in vivo* and its adhesion inhibitory properties in vitro point to pathological processes such as tumorigenesis and metastasis (Bode-Lesniewska *et al.*, Histol. & Cyto., 44, 303-312, 1996; Naso *et al,*. J. Biol. Chem., 269, 32999-33008, 1994).

Other CSPGs related to versican are brevican (Mr approximately 145 kDa) and neurocan (Mr > 300 kDa). Neither of these is known to be expressed by oligodendrocytes and are therefore not expected to be present in CNS myelin (Engel *et al.*, J. Comp. Neurol. 366, 34-43, 1996; Yamada *et al.*, J. Biol. Chem., 269, 10119-

10126, 1994).

Another CSPG family member that is not related to either versican or phosphacan, is NG2. Although it is expressed by O2A progenitor cells in the developing rat nervous system, it has no apparent homology to arretin-relevant GSPG's, and has an Mr approximately 400-800 kDa with a core protein of approximately 300 kDa (Nishiyama *et al.*, J. Cell Biol., 114, 359-371, 1991).

Neuroblastoma

Neuroblastoma arises from neuroectoderm and contains anaplastic sympathetic ganglion cells (reviewed in Pinkel and Howarth, 1985, In: Medical Oncology, Calabrese, P., Rosenberg, S. A., and Schein, P. S., eds., MacMillan, N.Y., pp. 1226-1257). One interesting aspect of neuroblastoma is that it has one of the highest rates of spontaneous regression among human tumors (Everson, 1964, Ann. N.Y. Acad. Sci. 114:721-735) and a correlation exists between such regression and maturation of benign ganglioneuroma (Bolande, 1977, Am. J. Dis. Child. 122:12-14). Neuroblastoma cells have been found to retain the capacity for morphological maturation in culture. The tumors may occur anywhere along the sympathetic chain, with 50% of such tumors originating in the adrenal medulla.

Neuroblastoma affects predominantly preschool aged children and is the most common extracranial solid tumor in childhood, constituting 6.5% of pediatric neoplasms. One half are less than two years of age upon diagnosis. Metastases are evident in 60% of the patients at presentation usually involving the bones, bone marrow, liver, or skin. The presenting symptoms may be related to the primary tumor (spinal coral compression, abdominal mass), metastatic tumor (bone pain) or metabolic effects of substances such as catecholamines or vasoactive polypeptides secreted by the tumor (e.g. hypertension, diarrhea). Experimental evidence indicates that an altered response to NGF is associated with neuroblastoma (Sonnenfeld and Ishii, 1982, J. Neurosci. Res.

8:375-391). NGF stimulated neurite outgrowth in one-half of the neuroblastoma cell lines tested; the other half was insensitive. However, NGF neither reduced the growth rate nor enhanced survival in any neuroblastoma cell line.Present therapies for neuroblastoma involve surgery and/or chemotherapy. Radiation therapy is used for incomplete tumor responses to chemotherapy. There is a 70-100% survival rate in individuals with localized tumors, but only a 20% survival rate in those with metastatic disease even with multiagent chemotherapy. It appears that patients less than one year have a better prognosis (70%) than older children.

This background information is provided for the purpose of making known information believed by the applicant to be of possible relevance to the present invention. No admission is necessarily intended, nor should be construed, that any of the preceding information constitutes prior art against the present invention. Publications referred to throughout the specification are hereby incorporated by reference in their entireties in this application.

SUMMARY OF THE INVENTION

The present invention relates to a neuron and neural tumor growth regulatory system, antibodies directed against the components of this system and diagnostic, therapeutic, and research uses for each of these aspects. The concept of a system is used to denote the functional relationship between the genes (for the regulatory factors and the receptors), their encoded protein-regulatory factors which regulate neuron growth (particularly neurite growth), and the receptors which are activated by the protein. The functional relationship allows one to use one component to identify and determine another. For example, having identified the protein component (factor or receptor), one can use techniques well known in the art to identify the gene.

In accordance with the present invention, a protein has now been identified, hereinafter

referred to as arretin, as one of the molecular components involved in contact-mediated growth inhibition on myelin. This protein has an apparent molecular weight of approximately 70 kDa, but it could be derived from a molecular complex. Given the purified protein, procedures for obtaining the other parts of the system are well known to those skilled in the art to purify the other components to the system. For example, the protein can be used in very standard techniques to obtain the amino acid sequence which can be used to obtain probes for nucleic acid sequences encoding arretin. Alternatively, arretin protein may be tagged for use as a reporter to detect receptors of arretin, which are then sequenced and used to obtain probes for the nucleic acid sequences encoding arretin receptors. Moreover, the production of antibodies to each of these components is also standard procedure.

The present invention further relates to arretin receptors and fragments thereof as well as the nucleic acid sequences coding for such arretin receptors and fragments, and their therapeutic and diagnostic uses. Substances which function as either agonists or antagonists to arretin receptors are also envisioned and within the scope of the present invention.

The present invention further relates to the nucleic acid sequences coding for arretin and its receptors, in addition to their therapeutic and diagnostic uses.

In accordance with another aspect of the present invention, there is provided the use of arretin for the regulation of growth of neurons and neural tumors.

In a further aspect of the present invention, there is provided a method for inhibiting growth of neural tumors, comprising the steps of introducing into the growth environment of the neurons a growth inhibiting amount of arretin, fragments thereof, or an arretin agonist.

In yet a further aspect of the present invention, arretin can be used to design small

molecules to block neurite outgrowth and neural tumor growth. These small molecules will be useful to block growth in situations involving aberrant sprouting, epilepsy, or metastasis.

A further embodiment involves a method of suppressing the inhibition of neuron growth, comprising the steps of delivering to the nerve growth environment, antibodies directed against arretin in an amount effective to reverse said inhibition.

In another aspect of the present invention arretin can be used to design antagonist agents that suppress the arretin-neuronal growth regulatory system. These antagonist agents can be used to promote axon regrowth and recovery from trauma or neurodegenerative disease.

In accordance with another aspect of the present invention, there is provided an assay method useful to identify arretin antagonist agents that suppress inhibition of neuron growth, comprising the steps of:

a) culturing neurons on a growth permissive substrate that incorporates a growth-inhibiting amount of arretin; and

b) exposing the cultured neurons of step a) to a candidate arretin antagonist agent in an amount and for a period sufficient prospectively to permit growth of the neurons;

thereby identifying as arretin antagonists the candidates of step b) which elicit neurite outgrowth from the cultured neurons of step a).

In yet another aspect of the present invention, there is provided an assay method useful for screening for compounds that stimulate cell adhesion and neurite growth, comprising the steps of:

a) coating a growth permissive substrate with a growth-inhibiting amount of arretin; and

b) adding a test compound and neuronal cells to the arretin-coated substrate;

c) washing to remove unattached cells;

d) measuring the viable cells attached to the substrate,

thereby identifying the cell adhesion candidates of step b) which elicit neurite outgrowth from the cultured neurons of step a).

In accordance with another aspect of the present invention, there is provided a method to suppress the inhibition of neurons, comprising the steps of delivering, to the nerve growth environment, an antagonist for arretin or its receptor in an amount effective to reverse said inhibition.

In another embodiment, the nucleic acids encoding arretin and/or its receptor can be used in antisense techniques and therapies.

Arretin inhibits neurite outgrowth in nerve cells and neuroblastoma cells. Such inhibitory protein comprises a 70,000 dalton molecular weight protein, aggregates, and analogs, derivatives, and fragments thereof. Arretin and its related proteins proteins may be used in the treatment of patients with malignant tumors which include but are not limited to melanoma and nerve tissue tumors (e.g., glioma, or neuroblastoma). The present invention also relates to antagonists of arretin, including, but not limited to, antibodies. Such antibodies can be used to neutralize the neurite growth inhibitory factors for regenerative repair after trauma, degeneration, or inflammation. In a further specific embodiment, monoclonal antibody may be used to promote regeneration of nerve fibers over long distances following spinal cord damage.

Various other objects and advantages of the present invention will become apparent from the detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Analysis of growth inhibition after separation of myelin proteins by DEAE

anion exchange chromatography.

A. Western blots of column fractions probed with anti-MAG antibody.

B. Neurite growth inhibition and protein profile present in the column fraction shown in A.

Figure 2. Identification of 70 kDa components in DEAE chromatographic fractions from CNS myelin as chondroitin sulphate proteoglycans. Myelin extracts (lane 1), DEAE chromatographic fractions 10, 25, and 32 (lanes 2, 3, and 4) were subjected to SDS-PAGE (6-16% acrylamide gradient) under reducing conditions, and detected by silver staining (A) and Western blots with anti-MAG (B), anti-TN-C (C), anti-TN-R (D), and anti-CS 473 antibodies (E). The position and molecular weight in kDa of marker proteins is indicated.

Figure 3. Western blot analysis of PNA affinity purification of the 70 kDa CSPGs from DEAE chromatographic fractions 20-34. A. Pooled DEAE chromatographic fractions 20-34 (lane 1), fractions 2 and 6 (lanes 2 and 3) of Hepes buffer wash, fractions 2 and 6 (lanes 4 and 5) of high salt buffer wash, and fractions 2, 4, 6, and 8 (lanes 6, 7, 8, and 9) were subjected to SDS-PAGE (6-16% acrylamide gradient) under reducing conditions, and detected by Western blots with anti-CS 473 antibody. B. Pooled DEAE chromatographic fractions 20-34 (lane 1), flow-through of PNA affinity column (lane 2), fraction 2 (lane 3) and pooled eluate (lane 4) were subjected to SDS-PAGE (6-16% acrylamide gradient) under reducing conditions, and detected by Western blots with anti-MAG antibodies. The position and molecular weight in kDa of marker proteins is indicated.

Figure 4. Identification of the 70 kDa components as phosphacan and versican-related molecules.

A and B. Western blot analysis with 3F8 polyclonal anti-phosphacan (A) and with polyclonal antibodies against recombinant versican (B). Fractions 20, 22, 24, 26, 28, 30, 32, and 34 (lanes 1-8) from DEAE chromatophy were subjected to SDS-PAGE

(6-16% acrylamide gradient) under reducing conditions. C, D, and E. Western blot analysis with 473 anti-CS antibody (C), 3F8 polyclonal anti-phosphacan (D) and polyclonal anti-recombinant versican (E). Myelin extracts (lane 1), pooled DEAE chromatographic fractions 20-34 (lane 2), pooled flow-through from the PNA affinity column (lane 3), and pooled eluates from PNA affinity column (lane 4) were subjected to SDS-PAGE (6-16% acrylamide gradient) as in A and B. The position and molecular weight in kDa of marker proteins is indicated.

Figure 5. Analysis of the 70 kDa CSPGs after chondroitinase ABC treatment. Pooled eluates from the PNA affinity column (lane 1) and chondroitinase ABC treated pooled eluates from PNA affinity column (lane 2) were subjected to SDS-PAGE (6-16% acrylamide gradient) under reducing conditions and detected by amido black staining (A) and by Western blots with polyclonal anti-phosphacan 3F8 (B). A bands at 28 kDa (in A. lane 1) is PNA (artifactually eluted). Two bands above 72 kDa (in A. lane 2) are chondrontinase ABC. The position and molecular weight in kDa of marker proteins is indicated.

Figure 6. Determination of cell-type expression of the 70 kDa CSPGs. Total membrane proteins (100 ┤ g) from brain (lane 1), myelin (lane 2), oligodendrocytes (lane 3), astrocytes (lane 4), cerebellar neurons (lane 5), hippocampal neurons (lane 6), NG 108-15 cells (lane 7), and L-cells (lane 8) were subjected to SDS-PAGE (6-16% acrylamide gradient) under reducing conditions and detected by Western blots with polyclonal anti-phosphacan 3F8. The position and molecular weight in kDa of marker proteins is indicated.

Figure 7. Inhibitory effects of the 70 kDa CSPGs on neurite outgrowth from cerebellar neurons.
Cerebellar neurons were plated as single cell suspensions on the 70 kDa CSPGs (arretin) and other substrates applied to PORN-treated nitrocellulose substrates. Cells were maintained for 24 h before fixation and staining with toluidine blue. Error bars

indicate standard deviation. Coating concentrations were about 50 nM (1:25 dilution) and 10 nM (1:125 dilution) for arretin and denatured arretin (DN) and 10 nM for laminin. Bars represent percent neurons with neurites (mean ± SD).

Figure 8. Inhibitory effects of the 70 kDa CSPGs on neurite outgrowth from hippocampal neurons. Hippocampal neurons were plated as single cell suspensions on the 70 kDa CSPGs (arretin) and other substrates applied to PORN-treated tissue culture plastic. Cells were maintained for 24 h before fixation and staining with toluidine blue. Error bars indicate standard deviation. Coating concentrations were about 50 nM (1:25 dilution) and 10 nM (1:125 dilution) for arretin and denatured arretin (DN) and 10 nM for laminin. Bars represent percent neurons with neurites (mean ± SD).

Figure 9. inhibitory effects of the 70 kDa CSPGs on neurite outgrowth from NG108-15 cells.
NG108 cells were plated as single cell suspensions on the 70 kDa CSPGs (arretin; inhib.p) and other substrates applied to PLL-treated tissue culture plastic. Cells were maintained for 24 h before fixation and staining with toluidine blue. Coating concentrations were about 50 nM (1:5 dilution) for arretin (inhib.p) and denature arretin (denat. inhib.p) and 10 nM for laminin (LM). Bars represent neurons with neurites (% growth). PLL= polylysine.

Figure 10. SDS-PAGE showing purification of arretin. The polypeptide was visualized by dyes after gel electrophoresis. Lane 1 shows arretin purified by peanut agglutinin (PNA) affinity chromatography. Two bands at approximately 70kDa are visible. A band at 28 kDa was identified as a peanut agglutinin contaminant. Lane 2 shows pooled fractions from a DEAE chromatographic column that were applied to the PNA column for further purification of the arretin bands. Lane 3 shows myelin starting material from which arretin was extracted. Lane 4 shows molecular weight markers.

Figure 11. Two-dimensional gel electrophoresis separation of arretin obtained from

PNA column chromatography. Polypeptides were separated in the first dimension by isoelectric focusing followed by SDS-PAGE separation according to size in the second dimension. Spots 1,2, and 3 at approximately 70 kDa are separated from each other by size and charge. The spot at 28 kDa is peanut agglutinin, verified by Western blotting (not shown).

Figure 12. Anti-arretin antibody 18D2 neutralizes neurite outgrowth inhibition and cell body repulsion by arretin on NG 108-15 cells. Picture A demonstrates cells growing normally on a substrate of arretin-polylysine overlaid with anti-arretin 18D2. Picture B shows cell growth is inhibited on a substrate of arretin-polylysine treated with control antiserum.

Figure 13. Western blot showing that culture supernatant from monoclonal antibody 18D2 recognizes the approximately 70 kDa arretin component. Lane 1 (arros) shows partially purified arretin. Lane 2 shows myelin. Lane 3 shows octylglucoside/salt extract of myelin.

Figure 14. Growth cone collapse by arretin. A. Collapsed growth cones (arrows) after addition of arretin. B. Growth cones treated with DMEM as a control remain spread. Explants of P2 rat dorsal root ganglion neurons were plated on laminin can cultured overnight to allow neurite extension. Arretin purified by lectin chromatography (A) or control medium (B) was added to the cultures. The cultures were fixed with paraformaldehyde 30 min. later and viewed by phase contrast microcopy. The numbers of collapsed growth cones were counted. Arretin caused significantly more growth cone collapse than the PBS or DMEM controls.

DETAILED DESCRIPTION OF THE INVENTION

For the purpose of the present invention the following terms are defined below.

The term, neurite growth regulatory factor, refers to either arretin or its receptor.

"Agonist" refers to a pharmaceutical agent having biological activity of inhibiting the neurite outgrowth of neurons cultured on a permissive substrate or inhibiting the regeneration of damaged neurons. It would be desirable to inhibit neuron growth in cases of epilepsy, neuroblastoma, and neuromas, a disease state in a mammal which includes neurite outgrowth or other neural growth of an abnormal sort which causes pain at the end of an amputated limb. Antagonists which may be used in accordance with the present invention include without limitation a arretin fragment, an analog of arretin of the arretin fragment, a derivative of either arretin, the arretin fragment or said analog, an anti-idiotypic arretin antibody or a binding fragment thereof, arretin ectodomain and a pharmaceutical agent.

"Antagonist" refers to a pharmaceutical agent which in accordance with the present invention which inhibits at least on biological activity normally associate with arretin, that is blocking or suppressing the inhibition of neuron growth. Antagonists which may be used in accordance with the present invention include without limitation a arretin antibody or a binding fragment of said antibody, a arretin fragment, a derivative of arretin or of a arretin fragment, an analog of arretin or of a arretin fragment or of said derivative, and a pharmaceutical agent, and is further characterized by the property of suppressing arretin-mediated inhibition of neurite outgrowth.

An arretin antagonist is therefore, a chemical compound possessing the ability to alter the biological activity of the neuronal receptor for arretin such that growth of neurons or their axons is suppressed. The agonist or antagonist of arretin in accordance with the present invention is not limited to arretin or its derivatives, but also includes the therapeutic application of all agents, referred herein as pharmaceutical agents, which alter the biological activity of the neuronal receptor for arretin such that growth of neurons or their axon is suppressed. The receptor can be identified with know technologies by those skilled in the art (Mason, (1994) *Curr. Biol.*, 4:1158-1161) and

its association with arretin or fragments thereof can be determined. The neuronal receptor for arretin may or may not be the same as cell surface molecules that recognize and bind arretin in an adhesion assay (Kelm et al., (1994) *Curr. Biol.*, 4:965-972). Once the active arretin-recognition domain of the receptor(s) is/are known, appropriate peptides or their analogs can be designed and prepared to serve as agonist or antagonist of the arretin-receptor interaction.

The term "effective amount" or "growth-inhibiting amount" refers to the amount of pharmaceutical agent required to produce a desired agonist or antagonist effect of the arretin biological activity. The precise effective amount will vary with the nature of pharmaceutical agent used and may be determined by one or ordinary skill in the art with only routine experimentation.

As used herein, the terms "arretin biological activity" refers to cellular events triggered by arretin, being of either biochemical or biophysical nature. The following list is provided, without limitation, which discloses some of the known activities associated with contact-mediated growth inhibition of neurite outgrowth, adhesion to neuronal cells, and promotion of neurite out growth from new born dorsal root ganglion neurons.

Use of the phrase "substantially pure" or "isolated" in the present specification and claims as a modifier of DNA, RNA, polypeptides or proteins means that the DNA, RNA, polypeptides or proteins so designated have been separated from their in vivo cellular environment. As a result of this separation and purification, the substantially pure DNAs, RNAs, polypeptides and proteins are useful in ways that the non-separated, impure DNAs, RNAs, polypeptides or proteins are not.

As used herein, the term "biologically active", or reference to the biological activity of arretin or, or polypeptide fragment thereof, refers to a polypeptide that is able to produce one of the functional characteristics exhibited by arretin or its receptors

described herein. In one embodiment, biologically active proteins are those that demonstrate inhibitory growth activities central nervous system neurons. Such activity may be assayed by any method known to those of skill in the art.

Based on the present evidence that arretin is a growth inhibitory protein in myelin, the means exist to identify agents and therapies that suppress arretin-mediated inhibition of nerve growth. Further, one can exploit the growth inhibiting properties of arretin, or arretin agonists, to suppress undesired nerve growth. Without the critical finding that arretin has growth inhibitory properties, these strategies would not be developed.

The description of the present invention comprising a neuron and neural tumor growth regulatory system can be divided into the following sections solely for the purpose of description: (1) isolation, purification and characterization of arretin; (2) production of arretin-related derivatives, analogs, and peptides; (3) arretin antagonists and assay methods to identify arretin antagonists; (4) characterization of arretin receptors; (5) molecular cloning of genes or gene fragments encoding arretin and its receptors; (6) generation of arretin related derivatives, analogs, and peptides; (7) production of antibodies against the components of the arretin growth regulatory system, (ie. arretin, its receptors, and the nucleic acid sequences coding for these proteins); (8) the diagnostic, therapeutic and research uses for each of these components and the antibodies directed thereto.

1. Isolation, Purification, and Characterization of Arretin

The present invention relates to CNS myelin associated inhibitory proteins of neurite growth and receptors of CNS myelin associated inhibitory proteins of neurite growth. The CNS myelin associated inhibitory proteins of the invention may be isolated by first isolating myelin and subsequent purification therefrom. Isolation procedures which may be employed are described more fully in the sections which follow. Alternatively, the CNS myelin associated inhibitory proteins may be obtained from a recombinant

expression system. Procedures for the isolation and purification of receptors for the CNS myelin associated inhibitory proteins are described below.

Isolation and Purification of Arretin Proteins

Arretin proteins can be isolated from the CNS myelin of higher vertebrates including, but not limited to, birds or mammals (both human and nonhuman such as bovine, rat, porcine, chick, etc.). Myelin can be obtained from the optic nerve or from central nervous system tissue that includes but is not limited to spinal cords or brain stems. The tissue may be homogenized using procedures described in the art (Colman et al., 1982, J. Cell Biol. 95:598-608). The myelin fraction can be isolated subsequently also using procedures described (Colman et al., 1982, supra).

In one embodiment of the invention, the CNS myelin associated inhibitory proteins can be solubilized in detergent (for e.g., see McKerracher et al., 1994). The solubilized proteins can subsequently be purified by various procedures known in the art, including but not limited to
chromatography (e.g., ion exchange, affinity, and sizing chromatography), centrifugation, electrophoretic procedures, differential solubility, or by any other standard technique for the purification of proteins. In one aspect, the solubilized proteins can be subjected to one or two-dimensional electrophoresis, followed by elution from the gel. Gel-eluted proteins can be further purified and/or used to generate antibodies.

Alternatively, the CNS myelin associated inhibitory proteins may be isolated and purified using immunological procedures. For example, in one embodiment of the invention, the proteins can first be solubilized using detergent. The proteins may then be isolated by immunoprecipitation with antibodies. Alternatively, the CNS myelin associated inhibitory proteins may be isolated using immunoaffinity chromatography in which the proteins are applied to an antibody column in

solubilized form.

2. Production of Arretin-Related Derivatives, Analogs, and Peptides

The production and use of derivatives, analogs, and peptides related to arretin are also envisioned, and within the scope of the present invention and include molecules antagonistic to neurite growth regulatory factors (for example, and not by way of limitation, anti-idiotype antibodies). Such derivatives, analogs, or peptides which have the desired inhibitory activity can be used, for example, in the treatment of neuroblastoma. Derivatives, analogs, or peptides related to a neurite growth regulatory factor can be tested for the desired activity by assays for nonpermissive substrate effects or for growth cone collapse.

The neurite growth regulatory factor-related derivatives, analogs, and peptides of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, a cloned neurite growth regulatory factor gene can be modified by any of numerous strategies known in the art (Maniatis, et al., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). A given neurite growth regulatory factor sequence can be cleaved at appropriate sites with restriction endonuclease(s), subjected to enzymatic modifications if desired, isolated, and ligated in vitro. In the production of a gene encoding a derivative, analogue, or peptide related to a neurite growth regulatory factor, care should be taken to ensure that the modified gene remains within the same translational reading frame as the neurite growth regulatory factor, uninterrupted by translational stop signals, in the gene region where the desired neurite growth regulatory factor-specific activity is encoded.

Additionally, a given neurite growth regulatory factor gene can be mutated in vitro or in vivo, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or

destroy preexisting ones, to facilitate further in vitro modification. Any technique for mutagenesis known in the art can be used, including but not limited to, in vitro site-directed mutagenesis (Hutchinson, et al., 1978, J. Biol. Chem. 253:6551), use of TAB.RTM. linkers (Pharmacia), etc.

3. Arretin Antagonists and Assay Methods to Identify Arretin Antagonists

In one embodiment suitable as arretin antagonist candidates are developed comprising fragments, analogs and derivatives of arretin. Such candidates may interfere with arretin-mediated growth inhibition as competitive but non-functional mimics of endogenous arretin. From the amino acid sequence of arretin and from the cloned DNA coding for it, it will be appreciated that arretin fragments can be produced either by peptide synthesis or by recombinant DNA expression of either a truncated domain of arretin, or of intact arretin could be prepared using standard recominant procedures, that can then be digested enzymically in either a random or a site-selective manner. Analogs of arretin or arretin fragments can be generated also by recombinant DNA techniques or by peptide synthesis, and will incorporate one or more, e.g. 1-5, L- or D-amino acid substitutions. Derivatives of arretin, arretin fragments and arretin analogs can be generated by chemical reaction of the parent substance to incorporate the desired derivatizing group, such as N-terminal, C-terminal and intra-residue modifying groups that have the effect of masking or stabilizing the substance or target amino acids within it.

In specific embodiments of the invention, candidate arretin antagonists include those that are derived from a determination of the functionally active region(s) of arretin. The antibodies mentioned above and any others to be prepared against epitopes in arretin, when found to be function-blocking in *in vitro* assays, can be used to map the active regions of the polypeptide as has been reported for other proteins (for example, see Fahrig et al., (1993) *Europ., J. Neurosci.,* 5: 1118-1126; Tropak et al., (1994) *J.*

Neurochem., 62: 854-862). Thus, it can be determined which regions of arretin are recognized by neuronal receptors and/or are involved in inhibition of neurite outgrowth. When those are known, synthetic peptides can be prepared to be assayed as candidate antagonists of the arretin effect. Derivatives of these can be prepared, including those with selected amino acid substitutions to provide desirable properties to enhance their effectiveness as antagonists of the arretin candidate functional regions of arretin can also be determined by the preparation of altered forms of the arretin domains using recombinant DNA technologies to produce deletion or insertion mutants that can be expressed in various cell types as chimaeric proteins that contain the Fc portion of immunoglobulin G (Kelm et al., (1994) *Curr. Biol.*, 4: 965-972). Alternatively, candidate mutant forms of arretin can be expressed on cell surfaces by transfection of various cultured cell types. All of the above forms of arretin, and forms that may be generated by technologies not limited to the above, can be tested for the presence of functional regions that inhibit or suppress neurite outgrowth, and can be used to design and prepare peptides to serve as antagonists.

In accordance with an aspect of the invention, the arretin antagonist is formulated as a pharmaceutical composition which contains the arretin antagonist in an amount effective to suppress arretin-mediated inhibition of nerve growth, in combination with a suitable pharmaceutical carrier. Such compositions are useful, in accordance with another aspect of the invention, to suppress arretin-inhibited nerve growth in patients diagnosed with a variety of neurological disorder, conditions and ailments of the PNS and the CNS where treatment to increase neurite extension, growth, or regeneration is desired, e.g., in patients with nervous system damage. Patients suffering from traumatic disorders (including but not limited to spinal cord injuries, spinal cord lesions, surgical nerve lesions or other CNS pathway lesions) damage secondary to infarction, infection, exposure to toxic agents, malignancy, paraneoplastic syndromes, or patients with various types of degenerative disorders of the central nervous system (Cutler, (1987) In: *Scientific American Medicines*, vol. 2, Scientific American Inc., N.Y., pp. 11-1-11-13) can be treated with such arretin antagonists. Examples of such

disorders include but are not limited to Strokes, Alzheimer's disease, Down's syndrome, Creutzfeldt-Jacob disease, kuru, Gerstman-Straussler syndrome, scrapie, transmissible mink encephalopathy, Huntington's disease, Riley-Day familial dysautonomia, multiple system atrophy, amylotrophic lateral sclerosis or Lou Gehrig's disease, progressive supranuclear palsy, Parkinson's disease and the like. The arretin antagonists may be used to promote the regeneration of CNS pathways, fiber systems and tracts. Administration of antibodies directed to an epitope of arretin, or the binding portion thereof, or cells secreting such antibodies can also be used to inhibit arretin function in patients. In a particular embodiment of the invention, the arretin antagonist is used to promote the regeneration of nerve fibers over long distances following spinal cord damage.

In another embodiment, the invention provides an assay method adapted to identify arretin antagonists, that is agents that block or suppress the growth-inhibiting action of arretin. In its most convenient form, the assay is a tissue culture assay that measures neurite out-growth as a convenient end-point, and accordingly uses nerve cells that extend neurites when grown on a permissive substrate. Nerve cells suitable in this regard include neuroblastoma cells of the NG108 lineage, such as NG108-15, as well as other neuronal cell lines such as PC12 cells (American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852 USA, ATCC accession NO. CRL 1721), human neuroblastoma cells, and primary cultures of CNS or PNS neurons taken from embryonic, postnatal or adult animals. The nerve cells, for instance about 10^3 cells-microwell or equivalent, are cultured on a growth permissive substrate, such as polylysine or laminin, that is over-layed with a growth-inhibiting amount of arretin. The arretin incorporated in the culture is suitably myelin-extracted arretin, although forms of arretin other than endogenous forms can be used provided they exhibit the arretin property of inhibiting neuron growth when added to a substrate that is otherwise growth permissive.

In this assay, candidate arretin antagonists, *i.e.*, compounds that block the growth-

inhibiting effect of arretin, are added to the arretin-containing tissue culture preferably in amount sufficient to neutralize the arretin growth-inhibiting activity, that is between 1.5 and 15 μg of arretin antagonists per well containing a density of 1000 NG108-15 cells/well cultured for 24 hr. in Dulbecco's minimal essential medium. After culturing for a period sufficient for neurite outgrowth, e.g. 3-7 days, the culture is evaluated for neurite outgrowth, and arretin antagonists are thereby revealed as those candidates which elicit neurite outgrowth. Desirably, candidates selected as arretin antagonists are those which elicit neurite outgrowth to a statistically significant extent compared to neurons plated on arretin alone.

Screening for compounds that stimulate cell adhesion and neurite growth on arretin-coated substrates.

Arretin not only prevents neurite growth but also reduces the adhesion of cells to the substrate. Since cell adhesion is technically far easier to assay quantitatively than neurite growth, cell adhesion can be used as a first screen for high-through-put screening of a large number of compounds. This can be done using the MTT [3{4-5-dimethylthiazol-2-yl]-2,5-diphenyltertrazolium bromide) assay. MTT is taken up by live cells and converted by the mitochondria into a blue substrate that can be quantified by a densitometer. For this assey, 96-well plates are coated with arretin. After washing wells the add chemical compounds can be added to the well for 1-2 hours or along with neuronal cells such as NG108-15 cells. After 2-4 hours or overnight incubation with the cells, the cultures are washed to remove unattached cells.
MTT is then added to the cells at a concentration of 0.5mg/ml in culture medium. Incubate for 4 hours at 37oC in a 5% CO_2 incubator. Wash once with PBS and add acid isopropanol (100ul/well), and mix with a pipette. After 5 minutes the plates are read with ELISA reader at 550nm.

Other assay tests that could be used include without limitation the following: 1) The growth cone collapse assay that is used to assess growth inhibitory activity of collapsin

(Raper, J.A., and Kapfhammer, J.P., (1990) *Neuron*, 2:21-29; Luo et al., (1993) *Cell*, 75:217-227) and of various other inhibitory molecules (Igarashi, M. et al., (1993) *Science*, 259:77-79) whereby the test substance is added to the culture medium and a loss of elaborate growth cone morphology is scored. 2) The use of patterned substrates to assess substrate preference (Walter, J. et al., (1987) *Development*, 101:909-913; Stahl et al., (1990) *Neuron*, 5:735-743) or avoidance of test substrates (Ethell, D.W. et al., (1993) *Dev. Brain Res.*, 72:1-8). 3) The expression of recombinant proteins on a heterologous cell surface, and the transfected cells are used in co-culture experiments. The ability of the neurons to extend neurites on the transfected cells is assessed (Mukhopadhyay et al., (1994) *Neuron*, 13:757-767). 4) The use of sections of tissue, such as sections of CNS white matter, to assess molecules that may modulate growth inhibition (Carbonetto et al., (1987) *J. Neuroscience*, 7:610-620; Savlo, T. and Schwab, M.E., (1989) *J. Neurosci.*, 9:1126-1133). 5) Neurite retraction assays whereby test substrates are applied to differentiated neural cells for their ability to induce or inhibit the retraction of previously extended neurites (Jalnink et al., (1994) *J. Cell Bio.*, 126:801-810; Sudan, H.S. et al., (1992) *Neuron*, 8:363-375; Smalheiser, N. (1993) *J. Neurochem.*, 61:340-342). 6) The repulsion of cell-cell interactions by cell aggregation assays (Kelm, S. et al., (1994) *Current Biology*, 4:965-972; Brady-Kainay, S. et al., (1993) *J. Cell Biol.*, 4:961-972). 7) The use of nitrocellulose to prepare substrates for growth assays to assess the ability of neural cells to extend neurites on the test substrate (Laganeur, C. and Lemmon, V., (1987)*PNAS*, 84:7753-7757; Dou, C-L and Levine, J.M., (1994) *J. Neuroscience*, 14:7616-7628).

Useful arretin antagonists include antibodies to arretin and the binding fragments of those antibodies. Antibodies which are either monoclonal or polyclonal can be produced which recognize arretin and its various epitopes using now routine procedures. For the raising of antibody, various host animals can be immunized by injection with arretin or fragment thereof, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and

incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinmitrophenol, and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin).

4. Isolation and Purification of Receptors for Arretin

Receptors for arretin can be isolated from cells whose attachment, spreading, growth and/or motility is inhibited by arretin. Such cells include but are not limited to fibroblasts and neurons. In a preferred embodiment, neurons are used as the source for isolation and purification of the receptors.

In one embodiment, receptors to arretin may be isolated by affinity chromatography of neuronal plasma membrane fractions, in which a myelin associated inhibitory protein or peptide fragment thereof is immobilized to a solid support. Alternatively, receptor cDNA may be isolated by expression cloning using purified arretin as a ligand for the selection of receptor-expressing clones.

Alternatively, arretin protein may be tagged for use as a reporter to detect receptors of arretin, using techniques that are well known in the art. There are many different types of tags that may be employed such as flourescence radioactive tags.

5. Molecular Cloning of Genes or Gene Fragments Encoding Arretin and Its Receptors

Any mammalian cell can potentially serve as the nucleic acid source for the molecular cloning of the genes encoding arretin or its receptors. The DNA may be obtained by standard procedures known in the art from cloned DNA (e.g., a DNA "library"), by chemical synthesis, by cDNA cloning, or by the cloning of genomic DNA, or fragments

thereof, purified from the desired mammalian cell. (See, for example, Maniatis et al., 1982, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.; Glover, D. M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U. K., Vol. I, II.) Clones derived from genomic DNA may contain regulatory and intron DNA regions, in addition to coding regions; clones derived from cDNA will contain only exon sequences. Whatever the source, a given neurite growth regulatory factor gene should be molecularly cloned into a suitable vector for propagation of the gene.

In the molecular cloning of a neurite growth regulatory factor gene from genomic DNA, DNA fragments are generated, some of which will encode the desired neurite growth regulatory factor gene. The DNA may be cleaved at specific sites using various restriction enzymes. Alternatively, one may use DNAse in the presence of manganese to fragment the DNA, or the DNA can be physically sheared, as for example, by sonication. The linear DNA fragments can then be separated according to size by standard techniques, including but not limited to, agarose and polyacrylamide gel electrophoresis and column chromatography.

Once the DNA fragments are generated, identification of the specific DNA fragment containing a neurite growth regulatory factor gene may be accomplished in a number of ways. For example, if an amount of a neurite growth regulatory factor gene or its specific RNA, or a fragment thereof, is available and can be purified and labeled, the generated DNA fragments may be screened by nucleic acid hybridization to the labeled probe (Benton and Davis, 1977, Science 196:180; Grunstein and Hogness, 1975, Proc. Natl. Acad. Sci. U.S.A. 72:3961-3965). For example, in a preferred embodiment, a portion of a neurite growth regulatory factor amino acid sequence can be used to deduce the DNA sequence, which DNA sequence can then be synthesized as an oligonucleotide for use as a hybridization probe. Alternatively, if a purified neurite growth regulatory factor probe is unavailable, nucleic acid fractions enriched in neurite growth regulatory factor may be used as a probe, as an initial selection procedure. It is

also possible to identify an appropriate neurite growth regulatory factor-encoding fragment by restriction enzyme digestion(s) and comparison of fragment sizes with those expected according to a known restriction map if such is available. Further selection on the basis of the properties of the gene, or the physical, chemical, or immunological properties of its expressed product, as described above, can be employed after the initial selection.

A neurite growth regulatory factor gene can also be identified by mRNA selection using nucleic acid hybridization followed by in vitro translation or translation in Xenopus oocytes. In an example of the latter procedure, oocytes are injected with total or size fractionated CNS mRNA populations, and the membrane-associated translation products are screened in a functional assay (3T3 cell spreading). Preadsorption of the RNA with complementary DNA (cDNA) pools leading to the absence of expressed inhibitory factors indicates the presence of the desired cDNA. Reduction of pool size will finally lead to isolation of a single cDNA clone. In an alternative procedure, DNA fragments can be used to isolate complementary mRNAs by hybridization. Such DNA fragments may represent available, purified neurite growth regulatory factor DNA, or DNA that has been enriched for neurite growth regulatory factor sequences. Immunoprecipitation analysis or functional assays of the in vitro translation products of the isolated mRNAs identifies the mRNA and, therefore, the cDNA fragments that contain neurite growth regulatory factor sequences. An example of such a functional assay involves an assay for nonpermissiveness in which the effect of the various translation products on the spreading of 3T3 cells on a polylysine coated tissue culture dish is observed. In addition, specific mRNAs may be selected by adsorption of polysomes isolated from cells to immobilized antibodies specifically directed against a neurite growth regulatory factor protein. A radiolabeled neurite growth regulatory factor cDNA can be synthesized using the selected mRNA (from the adsorbed polysomes) as a template. The radiolabeled mRNA or cDNA may then be used as a probe to identify the neurite growth regulatory factor DNA fragments from among other genomic DNA fragments.

Alternatives to isolating the neurite growth regulatory factor genomic DNA include, but are not limited to, chemically synthesizing the gene sequence itself from a known sequence or making cDNA to the mRNA which encodes the neurite growth regulatory factor gene. Other methods are possible and within the scope of the invention. The identified and isolated gene or cDNA can then be inserted into an appropriate cloning vector. A large number of vector-host systems known in the art may be used. Possible vectors include, but are not limited to, cosmids, plasmids or modified viruses, but the vector system must be compatible with the host cell used. Such vectors include, but are not limited to, bacteriophages such as lambda derivatives, or plasmids such as pBR322 or pUC plasmid derivatives. Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, etc.

In an alternative embodiment, the neurite growth regulatory factor gene may be identified and isolated after insertion into a suitable cloning vector, in a "shot gun" approach. Enrichment for a given neurite growth regulatory factor gene, for example, by size fractionation or subtraction of cDNA specific to low neurite growth regulatory factor producers, can be done before insertion into the cloning vector. In another embodiment, DNA may be inserted into an expression vector system, and the recombinant expression vector containing a neurite growth regulatory factor gene may then be detected by functional assays for the neurite growth regulatory factor protein.

The neurite growth regulatory factor gene is inserted into a cloning vector which can be used to transform, transfect, or infect appropriate host cells so that many copies of the gene sequences are generated. This can be accomplished by ligating the DNA fragment into a cloning vector which has complementary cohesive termini. However, if the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules may be enzymatically modified. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition

sequences. In an alternative method, the cleaved vector and neurite growth regulatory factor gene may be modified by homopolymeric tailing. Identification of the cloned neurite growth regulatory factor gene can be accomplished in a number of ways based on the properties of the DNA itself, or alternatively, on the physical, immunological, or functional properties of its encoded protein. For example, the DNA itself may be detected by plaque or colony nucleic acid hybridization to labeled probes (Benton, W. and Davis, R., 1977, Science 196:180; Grunstein, M. and Hogness, D., 1975, Proc. Natl. Acad. Sci. U.S.A. 72:3961). Alternatively, the presence of a neurite growth regulatory factor gene may be detected by assays based on properties of its expressed product. For example, cDNA clones, or DNA clones which hybrid-select the proper mRNAs, can be selected which produce a protein that inhibits in vitro neurite outgrowth. If an antibody to a neurite growth regulatory factor is available, a neurite growth regulatory factor protein may be identified by binding of labeled antibody to the putatively neurite growth regulatory factor-synthesizing clones, in an ELISA (enzyme-linked immunosorbent assay)-type procedure. In specific embodiments, transformation of host cells with recombinant DNA molecules that incorporate an isolated neurite growth regulatory factor gene, cDNA, or synthesized DNA sequence enables generation of multiple copies of the gene. Thus, the gene may be obtained in large quantities by growing transformants, isolating the recombinant DNA molecules from the transformants and, when necessary, retrieving the inserted gene from the isolated recombinant DNA. If the ultimate goal is to insert the gene into virus expression vectors such as vaccinia virus or adenovirus, the recombinant DNA molecule that incorporates a neurite growth regulatory factor gene can be modified so that the gene is flanked by virus sequences that allow for genetic recombination in cells infected with the virus so that the gene can be inserted into the viral genome. After the neurite growth regulatory factor DNA-containing clone has been identified, grown, and harvested, its DNA insert may be characterized as described herein. When the genetic structure of a neurite growth regulatory factor gene is known, it is possible to manipulate the structure for optimal use in the present invention. For example, promoter DNA may be ligated 5' of a neurite growth regulatory factor coding sequence,

in addition to or replacement of the native promoter to provide for increased expression of the protein. Many manipulations are possible, and within the scope of the present invention.

Expression of the Cloned Neurite Growth Regulatory Factor Genes.

The nucleotide sequence coding for a neurite growth regulatory factor protein or a portion thereof, can be inserted into an appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. The necessary transcriptional and translation signals can also be supplied by the native neurite growth regulatory factor gene and/or its flanking regions. A variety of host-vector systems may be utilized to express the protein-coding sequence. These include but are not limited to mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA. The expression elements of these vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used.Any of the methods previously described for the insertion of DNA fragments into a vector may be used to construct expression vectors containing a chimeric gene consisting of appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include in vitro recombinant DNA and synthetic techniques and in vivo recombinations (genetic recombination).

Expression vectors containing neurite growth regulatory factor gene inserts can be identified by three general approaches: (a) DNA-DNA hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a foreign gene inserted in an expression vector can be detected by DNA-DNA hybridization using probes comprising sequences that are

homologous to an inserted neurite growth regulatory factor gene. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign genes in the vector. For example, if a given neurite growth regulatory factor gene is inserted within the marker gene sequence of the vector, recombinants containing the neurite growth regulatory factor insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the foreign gene product expressed by the recombinant. Such assays can be based on the physical, immunological, or functional properties of a given neurite growth regulatory factor gene product.

Once a particular recombinant DNA molecule is identified and isolated, several methods known in the art may be used to propagate it. Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. As
previously explained, the expression vectors which can be used include, but are not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (e.g., lambda), and plasmid and cosmid DNA vectors, to name but a few.

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus, expression of the genetically engineered neurite growth regulatory factor protein may be controlled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (e.g., glycosylation, cleavage) of proteins. Appropriate cell lines or host

systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system can be used to produce an unglycosylated core protein product. Expression in yeast will produce a glycosylated product. Expression in mammalian (e.g. COS) cells can be used to ensure "native" glycosylation of the heterologous neurite growth regulatory factor protein. Furthermore, different vector/host expression systems may effect processing reactions such as proteolytic cleavages to different extents.

Identification and Purification of the Expressed Gene Product

Once a recombinant which expresses a given neurite growth regulatory factor gene is identified, the gene product can be purified and analyzed as described above. The amino acid sequence of arretin and its receptor protein can be deduced from the nucleotide sequence of the cloned gene, allowing the protein, or a fragment thereof, to be synthesized by standard chemical methods known in the art (e.g., see Hunkapiller, et al., 1984, Nature 310:105-111). In particular embodiments of the present invention, such neurite growth regulatory factor proteins, whether produced by recombinant DNA techniques or by chemical synthetic methods, include but are not limited to those containing altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amno acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine, and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Also included within the scope of the invention are neurite growth regulatory factor proteins

which are differentially modified during or after translation, e.g., by glycosylation, proteolytic cleavage, etc.

Characterization of the Neurite, Growth Regulatory Factor Genes

The structure of a given neurite growth regulatory factor gene can be analyzed by various methods known in the art.

The cloned DNA or cDNA corresponding to a given neurite growth regulatory factor gene can be analyzed by methods including but not limited to Southern hybridization (Southern, 1975, J. Mol. Biol. 98:503-517), Northern hybridization (Alwine, et al., 1977, Proc. Natl. Acad. Sci. U.S.A. 74:5350-5354; Wahl, et al., 1987, Meth. Enzymol. 152:572-581), restriction endonuclease mapping (Maniatis, et al., 1982, Molecular loning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), and DNA sequence analysis. DNA sequence analysis can be performed by any techniques known in the art including but not limited to the method of Maxam and Gilbert (1980, Meth. Enzymol. 65:499-560), the Sanger dideoxy method (Sanger, et al., 1977, Proc. Natl. Acad. Sci. U.S.A. 74:5463-5467), or use of an automated DNA sequenator (e.g., Applied Biosystems, Foster City, Calif.).

6. Production of Antibodies Against the Components of the Arretin Growth Regulatory System

Antibodies can be produced which recognize neurite growth regulatory factors or related proteins. Such antibodies can be polyclonal or monoclonal.Various procedures known in the art may be used for the production of polyclonal antibodies to epitopes of a given neurite growth regulatory factor. For the production of antibody, various host animals can be immunized by injection with a neurite growth regulatory factor protein, or a synthetic protein, or fragment thereof, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response,

depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum. A monoclonal antibody to an epitope of a neurite growth regulatory factor can be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein (1975, Nature 256:495-497), and the more recent human B cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72) and EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In a particular embodiment, the procedure described . may be used to obtain mouse monoclonal antibodies which recognize arretin and its receptors.

The monoclonal antibodies for therapeutic use may be human monoclonal antibodies or chimeric human-mouse (or other species) monoclonal antibodies. Human monoclonal antibodies may be made by any of numerous techniques known in the art (.RTM..q., Teng et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:7308-7312; Kozbor et al., 1983, Immunology Today 4:72-79; Olsson et al., 1982, Meth. Enzymol. 92:3-16). Chimeric antibody molecules may be prepared containing a mouse antigen-binding domain with human constant regions (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851, Takeda et al., 1985, Nature 314:452). A molecular clone of an antibody to a neurite growth regulatory factor epitope can be prepared by known techniques. Recombinant DNA methodology (see e.g., Maniatis et al., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) may be used to construct nucleic acid sequences which encode a monoclonal antibody olecule, or antigen binding region thereof.

A monoclonal antibody to an epitope of arretin can be prepared by using any technique

which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Köler and Milstein ((1975) *Nature*, 256:495-497), and the more recent human B cell hybridoma technique (Kozbor et al., (1983) *Immunology Today,* 4:72) and EBV-hybridoma technique (Cole et al., (1985) In *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp 77-96). In a particular embodiment, the procedure described by Nobile-Orazio et al. ((1984) *Neurology,* 34:1336-1342) may be used to obtain antibodies which recognize recombinant Arretin (for example of techniques, see Attia S. et al., (1993) *J. Neurochem.*, 61: 718-726).

The monoclonal antibodies for therapeutic use may be human monoclonal antibodies or chimeric human-mouse (or other species) monoclonal antibodies. Human monoclonal antibodies may be made by any of numerous techniques known in the art (e.g. Tan et al., (1983) *Proc. Natl. Acad. Sci. U.S.A.,* 80: 7308-7312; Kozbor et al., (1983) *Immunology Today, 4: 72-79; Olsson et al.,* (1982) *Meth. Enzymol.,* 92: 3-16*).* Chimeric antibody molecules may be prepared containing a mouse antigen-binding domain with human contact regions (Morrision et al., (1984) *Proc. Natl. Acad. Sci. U.S.A.,* 81: 6851; Takeda et al., (1985) *Nature,* 314: 452).

A molecular clone of an antibody to a Arretin epitope can be prepared by known techniques. Recombinant DNA methodology may be used to construct nucleic acid sequences which encode a monoclonal antibody molecule, or antigen binding region thereof (see e.g., Maniatis et al., (1982) In *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.).

For use, arretin antibody molecules may be purified by known techniques, such as immunoabsorption or immunoaffinity chromatography, chromotographic methods such as HPLC (high performance liquid chromatography), or a combination thereof, etc.

Arretin antibody fragments which contain the idiotype of the molecule can be generated

by known techniques. For example, such fragments include but are not limited to: the F (ab')$_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab, fragments which cen be generated by reducing the disulfide bridges of the F (ab')$_2$ fragment, and the two Fab or Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

Monoclonal antibodies known to react with human arretin may be tested for their usefulness to serve as arretin antagonists (Nobile-Orazio et al., (1984) *Neurology,* 34: 1336-1342; Doberson et al., (1985) *Neurochem. Res.,* 10: 499-513).

Antibody molecules may be purified by known techniques, e.g., immunoabsorption or immunoaffinity chromatography, chromatographic methods such as HPLC (high performance liquid chromatography), or a combination thereof, etc.Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the F(ab').sub.2 fragment which can be produced by pepsin digestion of the antibody molecule; the Fab, fragments which can be generated by reducing the disulfide bridges of the F(ab').sub.2 fragment, and the 2 Fab or Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

7. Diagnostic, Therapeutic and Research Uses for each of these Components and the Antibodies Directed Thereto

Arretin, its receptors, analogs, derivatives, and subsequences thereof, and anti-inhibitory protein antibodies or peptides have uses in diagnostics. Such molecules can be used in assays such as immunoassays to detect, prognose, diagnose, or monitor various conditions, diseases, and disorders affecting neurite growth extension, invasiveness, and regeneration. In one embodiment of the invention, these molecules may be used for the diagnosis of malignancies. Alternatively, the CNS myelin associated inhibitory proteins, analogs, derivatives, and subsequences thereof and

antibodies thereto may be used to monitor therapies for diseases and conditions which ultimately result in nerve damage; such diseases and conditions include but are not limited to CNS trauma, (e.g. spinal cord injuries), infarction, infection, malignancy, exposure to toxic agents, nutritional deficiency, paraneoplastic syndromes, and degenerative nerve diseases (including but not limited to Alzheimer's disease, Parkinson's disease, Huntington's Chorea, amyotrophic lateral sclerosis, progressive supra-nuclear palsy, and other dementias). In a specific embodiment, such molecules may be used to detect an increase in neurite outgrowth as an indicator of CNS fiber regeneration. For example, in specific embodiments, the absence of the CNS myelin associated inhibitory proteins in a patient sample containing CNS myelin can be a diagnostic marker for the presence of a malignancy, including but not limited to glioblastoma, neuroblastoma, and melanoma, or a condition involving nerve growth, invasiveness, or regeneration in a patient. In a particular embodiment, the absence of the inhibitory proteins can be detected by means of an immunoassay in which the lack of any binding to anti-inhibitory protein antibodies is observed. The immunoassays which can be used include but are not limited to competitive and non-competitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, precipitation reactions, gel diffusion precipitation reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, immunoelectrophoresis assays, and immunohistochemistry on tissue sections, to name but a few.

In accordance with another aspect of the invention, arretin and related compounds that retain the arretin property of inhibiting neurone growth (herein referred to as arretin agonists) are used therapeutically to treat conditions in which suppression of undesirable neuronal growth is desired. These include for example the treatment of tumors of nerve tissue and of conditions resulting from uncontrolled nerve sprouting such as is associated with epilepsy and in the spinal cord after nerve injury. In one embodiment patients with neuroblastoma, and particularly with neuropathies associated

with circulating arretin antibody, can be treated with arretin or arretin agonist.

Useful for nerve growth suppression are pharmaceutical compositions that contain, in an amount effective to suppress nerve growth, either arretin or a arretin agonist in combination with an acceptable carrier. Arretin can be obtained either by extraction from myelin as described above or, more practically, by recombinant DNA expression of Arretin-encoding DNA, for example, in the manner reported for MAG by Attia S., *et al.*, J. Neurochem.,61, 718-726, 1993. Useful arretin agonists are those compounds which, when added to the permissive substrate described above, suppress the growth of neuronal cells. Particularly useful Arretin agonists are those compounds which cause a statistically significant reduction in the number of neuronal cells that extend neurites, relative to control cells not exposed to the agonist. Candidate Arretin agonists include fragments of Arretin that incorporate the ectodomain, including the ectodomain *per se* and other N- and/or C-terminally truncated fragments of Arretin or the ectodomain, as well as analogs thereof in which amino acids, e.g. from 1 to 10 residues, are substituted, particularly conservatively, and derivatives of Arretin or Arretin fragments in which the N- and/or C-terminal residues are derivatized by chemical stabilizing groups. Such Arretin agonists can also include anti-idiotypes of Arretin antibodies and their binding fragments.

In specific embodiments of the invention, candidate Arretin agonists include specific regions of the Arretin molecule, and analogs or derivatives of these. These can be identified by using the same technologies described above for identification of Arretin regions that serve as inhibitors of neurite outgrowth.

The Arretin related derivatives, analogs, and fragments of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, Arretin-encoding DNA can be modified by any of numerous strategies known in the art (Maniatis *et al.*, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold

Spring Harbor, N.Y., 1982), such as by cleavage at appropriate sites with restriction endonuclease(s), subjected to enzymatic modifications if desired, isolated, and ligated *in-vitro*.

Additionally, the Arretin-encoding gene can be mutated *in-vitro* or *in-vivo* for instance in the manner applied fro production of the ectodomain, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in-vitro* modification. Any technique for mutagenesis known in the art cab be used, including but not limited to, *in-vitro* site directed mutagenesis (Hutchinson, *et al.*, J. Biol. Chem., 253, 6551, 1978), use of TAB™ linkers (Pharmacia), etc.

For delivery of Arretin, Arretin agonist or Arretin antagonist, various known delivery systems can be used, such as encapsulation in liposmes or semipermeable membranes, expression in suitably transformed or transfection glial cells, oligodendroglial cells, fibroblasts, etc. according to the procedure known to those skilled in the are (Lindvall *et al.*, Curr. Opinion Neurobiol., 4, 752-757, 1994). Linkage to ligands such as antibodies can be used to target delivery to myelin and to other therapeutically relevant sites *in-vivo*. Methods of introduction include, but are not limited to, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, oral, and intranasal routes, and transfusion into ventricles or a site of operation (e.g. for spinal cord lesions) or tumor removal. Likewise, cells secreting Arretin antagonist activity, for example, and not by way of limitation, hybridoma cells encapsulated in a suitable biological membrane may be implanted in a patient so as to provide a continuous source of Arretin inhibitor.

In another specific embodiment, ligands which bind to arretin or its receptors can be used in imaging techniques. For example, small peptides (e.g., inhibitory protein receptor fragments) which bind to the inhibitory proteins, and which are able to

penetrate through the blood-brain barrier, when labeled appropriately, can be used for imaging techniques such as PET (positron emission tomography) diagnosis or scintigraphy detection, under conditions noninvasive to the patient.

Neurite growth inhibitory factor genes, DNA, cDNA, and RNA, and related nucleic acid sequences and subsequences, including complementary sequences, can also be used in hybridization assays. The neurite growth inhibitory factor nucleic acid sequences, or subsequences thereof comprising about at least 15 nucleotides, can be used as hybridization probes. Hybridization assays can be used to detect, prognose, diagnose, or monitor conditions, disorders, or disease states associated with changes in neurite growth inhibitory factor expression as described supra. For example, total RNA in myelin, e.g., on biopsy tissue sections, from a patient can beassayed for the presence of neurite growth inhibitory factor mRNA, where the amount of neurite growth inhibitory factor mRNA is indicative of the level of inhibition of neurite outgrowth activity in a given patient.

Therapeutic Uses of Arretin

CNS myelin associated inhibitory proteins of the present invention can be therapeutically useful in the treatment of patients with malignant tumors including, but not limited to melanoma or tumors of nerve tissue (e.g. neuroblastoma). In one embodiment, patients with neuroblastoma can be treated with arretin or analogs, derivatives, or subsequences thereof, and the human functional equivalents thereof, which are inhibitors of neurite extension.

In an alternative embodiment, derivatives, analogs, or subsequences of CNS myelin inhibitory proteins which inhibit the native inhibitory protein function can be used in regimens where an increase in neurite extension, growth, or regeneration is desired, e.g., in patients with nervous system damage. Patients suffering from traumatic disorders (including but not limited to spinal cord injuries, spinal cord lesions, or other

CNS pathway lesions), surgical nerve lesions, damage secondary to infarction, infection, exposure to toxic agents, malignancy, paraneoplastic syndromes, or patients with various types of degenerative disorders of the central nervous system (Cutler, 1987, In: Scientific American Medicines v. 2, Scientific American Inc., N.Y., pp. 11-1-11-13) can be treated with such inhibitory protein antagonists. Examples of such disorders include but are not limited to Alzheimer's Disease, Parkinsons' Disease, Huntington's Chorea, amyotrophic lateral sclerosis, progressive supranuclear palsy and other dementias. Such antagonists may be used to promote the regeneration of CNS pathways, fiber systems and tracts. Administration of antibodies directed to an epitope of, (or the binding portion thereof, or cells secreting such as antibodies) can also be used to inhibit arretin protein function in patients. In a particular embodiment of the invention, antibodies directed to arretin may be used to promote the regeneration of nerve fibers over long distances following spinal cord damage.

Various delivery systems are known and can be used for delivery of arretin, related molecules, or antibodies thereto, e.g., encapsulation in liposomes or semipermeable membranes, expression by bacteria, etc. Linkage to ligands such as antibodies can be used to target myelin associated protein-related molecules to therapeutically desirable sites in vivo. Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, oral, and intranasal routes, and infusion into ventricles or a site of operation (e.g. for spinal cord lesions) or tumor removal. Likewise, cells secreting CNS myelin inhibitory protein antagonist activity, for example, and not by way of limitation, hybridoma cells, encapsulated in a suitable biological membrane may be implanted in a patient so as to provide a continuous source of anti-CNS myelin inhibiting protein antibodies.

In addition, any method which results in decreased synthesis of arretin or its receptors may be used to diminish their biological function. For example, and not by way of limitation, agents toxic to the cells which synthesize arretin and/or its receptors (e.g. oligodendrocytes) may be used to decrease the concentration of inhibitory proteins to

promote regeneration of neurons.

Arretin Receptors

Arretin receptors as well as analogs, derivatives, and subsequences thereof, and anti-receptor antibodies have uses in diagnostics. These molecules of the invention can be used in assays such as immunoassays or binding assays to detect, prognose, diagnose, or monitor various conditions, diseases, and disorders affecting neurite growth, extension, invasion, and regeneration. For example, it is possible that a lower level of expression of these receptors may be detected in various disorders associated with enhanced neurite sprouting and plasticity or regeneration such as those involving nerve damage, infarction, degenerative nerve diseases, or malignancies. The CNS myelin associated inhibitory protein receptors, analogs, derivatives, and subsequences thereof may also be used to monitor therapies for diseases and disorders which ultimately result in nerve damage, which include but are not limited to CNS trauma (e.g. spinal cord injuries), stroke, degenerative nerve diseases, and for malignancies.

The assays which can be used include but are not limited to those described above.

Arretin receptor genes and related nucleic acid sequences and subsequences, including complementary sequences, can also be used in hybridization assays, to detect, prognose, diagnose, or monitor conditions, disorders, or disease states associated with changes in neurite growth inhibitory factor receptor expression.

Arretin Receptors

Arretin receptors or fragments thereof, and antibodies thereto, can be therapeutically useful in the treatment of patients with nervous system damage including but not limited to that resulting from CNS trauma (e.g., spinal cord injuries), infarction, or degenerative disorders of the central nervous system which include but are not limited

to Alzheimer's disease, Parkinson's disease, Huntington's Chorea, amyotrophic lateral sclerosis, or progressive supranuclear palsy. For example, in one embodiment, arretin receptors, or subsequences or analogs thereof which contain the inhibitory protein binding site, can be administered to a patient to "compete out" binding of the inhibitory proteins to their natural receptor, and to thus promote nerve growth or regeneration in the patient. In an alternative embodiment, antibodies to the inhibitory protein receptor (or the binding portion thereof or cells secreting antibodies binding to the receptor) can be administered to a patient in order to prevent receptor function and thus promote nerve growth or regeneration in the patient. Patients in whom such a therapy may be desired include but are not limited to those with nerve damage, stroke, or degenerative disorders of the central nervous system as described supra.

Various delivery systems are known and can be used for delivery of arretin receptors, related molecules, or antibodies thereto, e.g., encapsulation in liposomes, expression by bacteria, etc. Linkage to ligands such as antibodies can be used to target arretin-related molecules to therapeutically desirable sites in vivo. Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, oral, intranasal routes, and infusion into ventricles or a site of tumor removal.

The present invention is directed to genes and their encoded proteins which regulate neurite growth and the diagnostic and therapeutic uses of such proteins. The proteins of the present invention (arretin and its receptors) include proteins associated with central nervous system myelin with highly nonpermissive substrate properties, termed herein neurite growth inhibitory factors.

The present invention is also directed to antibodies to and peptide fragments and derivatives of the neurite growth inhibitory proteins and their therapeutic and diagnostic uses. These antibodies or peptides can be used in the treatment of nerve damage resulting from, e.g., trauma (e.g., spinal cord injuries), stroke, degenerative

disorders of the central nervous system, etc. In particular, antibodies to arretin proteins may be used to promote regeneration of nerve fibers. In a specific embodiment of the invention, monoclonal antibodies directed to arretin and/or its receptors may be used to promote the regeneration of nerve fibers over long distances following spinal cord damage.

The present invention is described in further detail in the following non-limiting examples. It is to be understood that the examples described below are not meant to limit the scope of the present invention. It is expected that numerous variants will be obvious to the person skilled in the art to which the present invention pertains, without any departure from the spirit of the present invention. The appended claims, properly construed, form the only limitation upon the scope of the present invention.

EXAMPLES

Example I: Isolation and characterization of a novel neurite growth inhibitory molecule from mammalian central nervous system myelin

Animals.

ICR mice and Wistar rat embryos were obtained from the animal facilities at Charles River.

Materials.

The following lectins were purchased from Sigma: Maclura pomifera (osage orange), Arachis hypogaea (PNA), Ulex europaeus (gorse), Phaseolus vulgaris PHA-L (red kidney bean), Triticum vulgaris (wheat germ), and Concanavalin A (jack bean). Laminin from EHS sarcoma, Poly-L-ornithine (PORN), Poly-L-lysine (PLL), Chondroitinase ABC (chondroitin ABC lyase, E.C. 4.2.2.4. from Proteus vulgaris,

protease-free), heparinase and PNA agrose beads were also purchased from Sigma. Horseradish peroxidase (HRP)- conjugated secondary antibodies to rabbit, rat or mouse IgG and IgM were purchased from Amersham and Jackson Labs.

Antibodies.

Monoclonal antibody 473-HD is a mouse IgM against a chondroitin sulphate epitope on mouse brain proteoglycans (Faissner *et al.*, J. Cell Biol., 126, 783-799, 1994). Rabbit polyclonal anti-versican antibodies were generated against recombinantly expressed human versican fusion proteins. We used monoclonal anti-L2 antibody (412) from rat (Kruse *et al.*, Nature, 316, 146-148, 1985) and polyclonal antibody 3F8 against phosphacan (Engel *et al.*, J. Comp. Neurol., 366, 34-43, 1996; Meyer-Puttlitz *et al.*, J. Comp. Neurol., 366, 44-54, 1996).

Multiple neurite growth inhibitory activities are present in extracts of CNS myelin after DEAE chromatography. We have previously shown that two peaks of neurite growth inhibitory activity are present in fractions of myelin extracts following DEAE chromatography (McKerracher *et al.*, Neuron, 13, 805-811, 1994). The largest of these peaks is associated with the earlier fractions eluted off the DEAE column by a 0.2 to 2 M gradient. A substantial proportion of the inhibitory activity in this peak is associated with myelin-associated glycoprotein (MAG). The inhibitory activity in column fractions was assayed by an in vitro bioassay using a neuronal cell line (NG108-15). These results suggest that molecule(s) other than MAG also contribute to the inhibitory activity associated with CNS myelin (Fig. 1).

Identification of a 70k Da protein associated with CNS myelin

In addition to MAG and the NI35/250 inhibitory molecules associated with myelin (McKerracher,

et al., 1994; Mukhopadhyay *et al.*, Neuron, 13, 757-767, 1994; Schwab *et al.*, Ann. Rev. Neurosci., 16, 565-595, 1993), three extracellular matrix, molecules namely, tenascin-C (TN-C), tenascin-R (TN-R) and chondroitin sulfate proteoglycans (CSPGs)

that are distributed in many CNS and non-CNS tissues are also known to have neurite growth inhibitory activity (Schachner *et al.*, 1994). We therefore investigated which of these inhibitory molecules are found in the two inhibitory peaks obtained after DEAE chromatography of CNS myelin extracts. DEAE column chromatographic fractions that contained the first (fractions 10) and second (fraction 26) inhibitory peaks were subjected to SDS-PAGE on a 6-16% polyacrylamide gradient gel under reducing conditions. These gels were either silver stained (Fig. 2A) or Western blotted with anti-MAG, TN-C, TN-R, and a monoclonal antibody against chondroitin sulfate (mAb 473) (Fig. 2B-E). The silver stained gels (2A) showed any bands. Anti-MAG antibody recognizes a 100 kDa band that is highly enriched in fraction10 but is much weaker in fractions 26 and 32 (Fig 2B). The intensity of the 200 and 220 kDa bands labelled with anti-TN-C was similar to that of the MAG antibody, i.e., enriched in fraction 10 (Fig. 2C). However, the 160 and 180 kDa bands recognized by the anti-TN-R antibody were present only in the total myelin extract and in fraction 10 (Fig. 2D). Interestingly, the anti-CS mAb 473 recognized 70 kDa band and a slightly small minor band in fractions 26 and 32 but not in the octylglucoside extract of myelin and/or in fraction 10. This shows that these components can only be detected immunochemically after substantial enrichment during the purification steps. These experiments show that MAG, TN-C and TN-R may contribute to the inhibitory effects of the first peak, and that MAG, TN-C and the 70 kDa bands may contribute to the second inhibitory peak. Western blots of samples of brain membranes probed with mab 412 that recognizes the HNK-1 epitope indicates that this carbohydrate epitope is not found in the 70 kDa components (data not shown).

Enzymatic hydrolysis with chondroitinase ABC and heparinase

Proteins were treated with chondroitinase ABC (0.02 U/ml) in 50 mM Tris-acetate (pH 8.0) for 2.5 h at 37°c in the presence of protease inhibitors (5 mM benzamidine, 1 mM iodoacetamide and 5 mM p-tosyl-L-lysine chloromethyl ketone, sodium salt). Heparinase digestion was done according to the manufacturer's instructions.

Purification of Arretin.

Preparation of myelin extracts and their fractionation by DEAE chromatography have been described (McKerracher *et al.,* 1994; see Fig.1). For further purification by lectin affinity chromatography, PNA-conjugated agarose beads (1.2 ml) were used. DEAE chromatographic fractions number 20 to 34 (2 ml each) were pooled (about 30 ml), diluted with 3 volume of H_2O, and loaded on the PNA-agarose column. The flow-through was reloaded three times, and the column was subsequently washed with 12 ml Hepes buffer (pH7.5, 0.08% Sodium azide, 10 mM Hepes, 0.15 mM NaCl, 0.1 mM Ca^{2+}, and 0.01 mM Mn^{2+}), followed by 12 ml of a high salt buffer (pH7.5, 2 M NaCl, and 20 mM Triethanolamine). The column was eluted with 20 ml of elution buffer (2 M NaCl, 20 mM Trithanolamine, pH7.5, and 0.5 M D-galactose). Appropriately pooled fractions were dialysed against 1000 ml of H_2O at 40°C, lyophilised, and dissolved in 1 ml of H_2O, such that the final concentration was about 0.16 M NaCl, 1.6 mM Trithanolamine, pH7.5, and 0.04 M D-galactose. Samples were aliquoted, and stored at -70°c. The protein profile was determined by SDS-PAGE on gradient gels (6 to 16% polyacrylamide) (Laemmli, U.K., Nature, 277, 680-685, 1970), by two-dimensional electrophorens and by Western blots (Towbin *et al.,* Proc. Nat, Acad. Sci. USA., 76, 4350-4354, 1979). Protein concentrations were estimated according to Bradford (1976).

Reactivity of Arretin with lectins.

Proteins tranferred to membranes were blocked with 2% bovine serum albumin (BAS) in TBS buffer (20 mM Tris-HCl, 500 mM NaCl, pH7.5) for 1 h, and incubated separately with 6g/ml of different HRP- or biotin-conjugated lectins for 2 h. The membranes were washed with TTBS (20 Mm Tris-HCl, 500 mM NaCl, 0.05% Tween-20, pH7.5) for 1h and complexes were detected by ECL (DU PONT) or the AP-ABC (VECTOR) Kit according to the manufacturer's instructions. As positive controls for lectin binding, several sugars, including galactose, glucose, glucosamine, galactosamine, fucose, and mannose (at 20 mg/ml), were applied as spots on nitrocellulose.

Purification by Lectin affinity chromatography

To further purify the 70 kDa components from DEAE fractions containing the second inhibitory peak, we screened the ability of the components to bind the following lectins: Maclura pomifera (osage orange), Arachis hypogaea (PNA), Ulex europaeus uea I (gorse or furze). Phaseolus vulgaris (PHA-L), Triticum vulgaris (wheatgerm agglutinin) and Concanavalin A (Con-A). Nitrocellulose membranes electro blotted with pooled DEAE fractions 20 to26 after protein separation by SDS-PAGE were probed with the various lectins. All the lectins except Con-A bound only to the 70 kDa bands (not shown).

We next tested whether the 70 kDa components could be purified by binding to lectin. For this, PNA-conjugated agarose beads were chosen. Fractions 20 to 26 obtained from DEAE column chromatography of bovine CNS myelin extracts were pooled and incubated with PNA-conjugated beads in an Eppendorf tube. After washing the beads, the proteins bound to the PNA-beads were separated by SDS-PAGE, electrophoretically blotted onto nitrocellulose membrane and probed with anti-MAG, TN-C and the 473 antibodies. As expected only the 70 kDa bands were recognized by the mAb 473. No labeling was observed with the other two antibodies, indicating that PNA lectin can be used to separate the 80 kDa molecule from MAG and TN-C (not shown).

A two-step purification of the 70 kDa components was therefore attempted. Octylglucoside extracts of bovine CNS myelin were passed though a DEAE column, and the material eluted by a NaCl gradient, and fractions 20-34 were pooled. The pooled fractions were then subjected to PNA-affinity chromatography. The material eluted from the PNA column was separated on a SDS-PAGE gradient gel (6-16%

acrylamide) under reducing conditions. The gels were then stained with silver, or Western blotted and probed with anti-MAG, TN-C and 473 antibodies. A 70 kDa doublet was seen after Amido black staining (Fig. 5A). This major band was recognized only by the 473 anti-CS antibody (Fig. 3A), but not by anti-MAG (Fig. 3B) or anti-TN-C antibodies (not shown). The minor component just below the major band was not visible in this preparation.

The 70 kDa components are novel phosphocan-versican-related molecules. We further investigated whether the 70 kDa bands purified from CNS myelin shared epitopes with other known CSPGs. On Western blots of the DEAE chromatographic fractions the 70 kDa bands also reacted with polyclonal antibodies against phosphacan and recombinant versican (Fig. 4A and B). Both these antibodies plus the 473 anti-CS recognized the 70 kDa PNA affinity purified polypeptides (Fig. 4C, D, E). After chondroitinase ABC treatment, the major 70 kDa proteins were found to have an apparent Mr of 50 kDa (Fig. 5A) which did not react with the anti-CS mAb 473 (not shown), but did react with anti-phosphacan (Fig. 5B) and anti-versican. Since native phosphacan has a molecular weight of 500-600 kDa (core protein 400 kDa), and versican is a very large proteoglycan with a molecular weight of 900 kDa (core protein 400 kDa), the 70 kDa components that we have isolated from CNS myelin are likely to be novel proteins. We call these proteins arretin (collectively). The 2 bands may represent 2 isoforms, or the smaller component may be an altered version of the larger, due to degradation.

The 70 kDa proteins inhibit neurite growth. The present invention involved a test that examined effects of the 70 kDa myelin-derived proteins in modulating neurite growth from rat hippocampal and cerebellar granule celleurons. The 70 kDa proteins inhibited neurite growth from neonatal rat cerebellar and hippocampal neurons (Figs. 7 and 8), as well as from cultured NG108-15 cells (Fig. 9). This inhibitory activity was lost after heat denaturation. These result indicate that novel myelin-associated 70 kDa proteins are inhibitors of neurite growth, and are likely to be largely responsible for the activity associated with the second inhibitory peak in fractions obtained after DEAE separation

of CNS myelin extracts. The present invention comprises these new inhibitors collectively termed as arretin.

Assays for repulsion of growth cones and cell bodies.

Tissue culture dishes (Becton Dickinson) with 24 wells were coated with methanol-solubilized nitrocellulose according to Lagenaur and Lemmon (1987) and air-dried in a sterile hood. For assays addressing the effect of arretin on growth cones, nitrocellulose and poly-L-lysine (PLL 0.01%) coated dishes were used as described (Xiao *et al.*, Neurosci., 8, 766-782, 1996). The dishes were washed three times with PBS and dried in a sterile hood. Different test proteins (arretin, denatured (80°c for 30 min) arretin, TN-R, and laminin), each at concentrations of 2 nM, 10nM, and 50nM, were applied in duplicate as 2.5 µl single spots to the dishes and incubated overnight at 37°c in a humidified atmosphere.

Determination of substrate coating efficiency was been described by Xiao et al., 1996. Before plating the NG108 cells or cerebellar neurons, the dishes were washed with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (CMF-HBSS). Explants were prepared from cerebella of 6 to 7-day-old mice and maintained in a chemically defined medium (Fischer *et al.,* J. Neurosci., 6, 605-612, 1986; Fischer, G., Neurosci. Lett., 28, 325-329, 1982). Explants were allowed to grow neurites for 72 h and then fixed with glutaraldehyde in PBS at a final concentration of 2.5%.

After fixation, cultures were stained with 0.5% toluidine blue (Sigma) in 2.5% sodium carbonate, washed five times with water and air dried. All experiments were performed at least three times. Assay for neurite outgrowth. Hippocampal neurons from 18- to 19-day-old rat embryos were prepared as described (Keilhauer *et al.*, Nature, 316, 728-730, 1985; Lochter *et al.*, J. Cell Biol., 113, 1159-1171, 1991; Dorries *et al.*, 1995 ?). For the assays on neurite outgrowth, hippocampal neurons were maintained in chemically defined medium (Rousselet *et al.*, Ann. Rev. Cell Biol., 129, 495-504, 1988; Lochter and Schachner, J. Neurosci., 13, 3986-4000, 1993; Xiao *et al.*, 1996).

Briefly, 96-well plates (Nunc) were pretreated with 5g/ml poly-L-ornithine (PORN) for 1 to 2 hours at 37°c, washed twice with water and air-dried. Proteins at concentrations of 2 nM, 10 nM, and 50 nM were coated on the dried surfaces overnight at 37°c in a humidified atmosphere. Determination of substrate coating efficiency as described Xiao *et al.*, 1996. The plates were washed three times with CMF-HBSS and hippocampal neurons prepared from 18- to 19-day-old rat embryos (Keilhauer *et al.*, 1985; Lochter *et al.*, 1991; Dorries *et al.*, 1995) were plated at a density of 3,000 cells per well in 100µl a chemically defined medium (Rousselet *et al.*, 1988; Lochter and Schachner, 1993; Xiao *et al.*, 1996). After 12 h, cells were fixed without a preceding washing step by gentle addition of 25% glutaraldehyde to a final concentration of 2.5%. After fixation, cultures were stained with toluidine blue and morphological parameters were quantified with an IBAS image analysis system. For morphometric analysis, only cells without contact with other cells were evaluated. Neurites were defined as those processes with a length of at least one cell body diameter. The total neurite length per cell was determined by analysing 50 cells in each of two wells. To determine the number of cells with neurites, 100 neurons in each of two wells were counted per experiment. Raw data from at least three independent experiments were analyzed by ANOVA and by the Newman-Keuls test with $P < 0.05$ and $P < 0.01$ being considered significant or highly significant, respectively. All graphs comprise data derived from at least three independent experiments.

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications to the invention to adapt it to various usages and conditions. Such changes and modifications are properly, equitably, and intended to be within the full range of equivalence of the following claims.

CLAIMS

1. A protein consisting of a molecule, derivative or fragment thereof, characterized by the following properties:
   a) said protein has an apparent molecular weight of 70 kDa; and
   b) said protein mediates inhibition of neurite outgrowth.

2. A nucleic acid sequence encoding the protein, derivative, or fragment thereof as in claim 1.

3. An isolated receptor that binds the protein of claim 1.

4. A nucleic acid sequence encoding the receptor, derivative, or fragment thereof as in claim 3.

5. An antagonist comprising an antibody or a binding fragment thereof, directed toward the protein, derivative or fragment of claim 1.

6. A fragment, analog or derivative of the protein of claim 1, which interferes with arretin mediated inhibition as competitive but non-functional mimics of endogenous arretin.

7. An antagonist comprising a peptide or its analog modelled on a sequence of the protein of claim 1 which serves as an antagonist of the arretin-receptor interaction.

8. An antagonist comprising blocking peptides or small molecules modelled on an extracellular region of the protein of claim 1 which mediates inhibitory activity.

9. An antagonist compromising a peptide, peptidiomimetic compound, or derivative thereof that is capable of neutralizing the inhibitory substrate property of the protein of claim 1 which said neutralization is detected by

observing the ability of said antagonist to suppress inhibition of neuron growth, comprising the steps of:

a) culturing neurons on a growth substrate that incorporates a growth-inhibiting amount of arretin; and

b) exposing said cultured neurons to said antagonist agent in an amount and for a period sufficient prospectively to permit growth of said neurons.

10. An antagonist compromising a peptide, peptidiomimetic compound, or derivative thereof that is capable of neutralizing the inhibitory substrate property of the protein of claim 1, said neutralization detected by observing the ability of said antagonist to suppress inhibition of neuron growth, comprising the steps of:

a) culturing neurons on a growth substrate that incorporates a growth-inhibiting amount of arretin; and

b) exposing said cultured neurons to said antagonist agent in an amount and for a period sufficient prospectively to block the growth cone collapse response by arretin.

11. An antagonist comprising a chemical compound possessing the ability to alter the biological activity of the neuronal receptor for the protein of claim 1 such that growth of neurons or their axons is suppressed.

12. An isolated and purified antibody or binding fragment thereof, capable of neutralizing the biological activity of the protein of claim 1, wherein the antibody is a monoclonal antibody.

13. A hybridoma cell line which produces the monoclonal antibody of claim 12.

14. An isolated and purified antibody or binding fragment thereof, capable of

neutralizing the biological activity of the protein of claim 1, wherein the antibody is a polyclonal antibody.

15. The use of the protein of claim 1, biologically active variants or fragments thereof, for raising antibodies or ligands thereof which overcome growth inhibition.

16. A polypeptide having an amino acid sequence or a subsequence thereof wherein the polypeptide has from about 18 to 23 amino acid residues such that antibodies having antagonistic activity to the protein of claim 1 can be raised against said polypeptide.

17. A hybridoma cell line producing an antibody that specifically binds arretin.

18. A pharmaceutical composition comprising an antibody having the property of inhibiting arretin activity wherein arretin has an apparent molecular weight of 70 kDa, wherein the antibody of arretin is isolated from the blood serum of an animal to which said arretin has been previously added.

19. An arretin antagonist formulated as a pharmaceutical composition containing one or more arretin antagonists in an amount effective to suppress arretin-mediated inhibition of nerve growth, in combination with a suitable pharmaceutical carrier, wherein said antagonist is selected from the group comprising a fragment of arretin, a peptide, or a chemical molecule.

20. A pharmaceutical composition for nerve regeneration treatment of a patient comprising an effective amount of an arretin antagonist in a suitable pharmacologic carrier.

21. A pharmaceutical composition for treatment of a patient with damage to the

central nervous system comprising an effective amount of a substance that is capable of neutralizing the inhibitory substrate property of arretin in which neutralization is detected by observing the ability of the antagonist to suppress inhibition of neuron growth, comprising the steps of:

a) culturing neurons on a growth substrate that incorporates a growth-inhibiting amount of arretin; and

b) exposing said cultured neurons to the arretin antagonist agent in an amount and for a period sufficient prospectively to permit growth of said neurons.

22. The pharmaceutical composition of claim 21, wherein the antagonist substance is an antibody or binding region thereof.

23. The pharmaceutical composition of claim 21, wherein the damage is due to infarction, traumatic injury, surgical lesion or a degenerative disorder of the central nervous system.

24. The pharmaceutical composition of claim 21, wherein the damage has occurred to the spinal cord.

25. The pharmaceutical composition of claim 21, wherein the antibody is administered by the introduction into the patient of an antibody-secreting cell.

26. A pharmaceutical composition for treatment of a patient with damage to the central nervous system or the peripheral nervous system comprising an effective amount of arretin antagonist consisting of a peptide, peptidiomimetic compound, or derivative thereof that is capable of neutralizing the inhibitory substrate property of arretin in which neutralization is detected by observing the ability of the antagonist to suppress inhibition of neuron growth, comprising the steps of:

a) culturing neurons on a growth permissive substrate that incorporates a growth-inhibiting amount of arretin; and

b) exposing said cultured neurons to the arretin antagonist agent in an amount and for a period sufficient prospectively to permit growth of said neurons.

27. A method effective to suppress the inhibition of neuron growth, comprising the step of delivering an arretin antagonist to the nerve growth environment in an amount effective to reverse said inhibition.

28. A method according to claim 27, wherein said arretin antagonist is selected from an arretin antibody or a binding fragment of said antibody, an arretin fragment, a derivative of an arretin fragment, an analog of arretin or of an arretin fragment or of said derivative, and a pharmaceutical agent, and is further characterized by the property of suppressing arretin-mediated inhibition of neurite outgrowth.

29. A method according to claim 28, wherein said arretin antagonist is an arretin antibody or a binding fragment thereof.

30. A method according to claim 27, 28, or 29, wherein said arretin antagonist is delivered to the growth environment of a CNS neuron requiring growth or regeneration as a result of spinal cord injury, spinal cord lesions, surgical nerve lesions, damage secondary to infarction, infection, exposure to toxic agents and malignancy.

31. A method according to claims 27, 28, or 29, wherein said arretin antagonist is delivered to a patient having a medical condition selected from Strokes, Alzheimer's disease, Down's syndrome, Creutzfeldt-Jacob disease, kuru, Gerstman-Straussler syndrome, scrapie, transmissible mink encephalopathy,

Huntington's disease, Riley-Day familial dysautonomia, multiple system atrophy, amyotropic lateral sclerosis or Lou Gehrig's disease, progressive supranuclear palsy, Parkinson's disease.

32. The use of the antagonist of claim 18 to treat a patient with damage to the central nervous system comprising administering to the patient an effective amount of monoclonal antibody directed towards arretin, wherein arretin has an apparent molecular weight of 70 kDa, and said antibody blocks the inhibitory effects of arretin, or a fragment thereof containing the binding region.

33. An assay method useful to identify arretin antagonist agents that suppress inhibition of neuron growth, comprising the steps of:

a) culturing neurons on a growth substrate that incorporates a growth-inhibiting amount of arretin; and

b) exposing the cultured neurons of step a) to a candidate arretin antagonist agent in an amount and for a period sufficient prospectively to permit growth of said neurons;

thereby identifying as arretin antagonists said candidates of step b) which elicit neurite outgrowth from said cultured neurons of step a)

34. An assay method as in claim 33, wherein the cultured neurons are selected from the group comprising primary neurons or neuronal cell lines.

35. A method for screening for compounds that stimulate cell adhesion and neurite growth, comprising the steps of:

e) coating a growth permissive substrate with a growth-inhibiting amount of arretin;

f) adding a test compound and neuronal cells to the arretin-coated substrate;

g) washing to remove unattached cells; and

h) measuring the viable cells attached to the substrate,

thereby identifying the cell adhesion candidates of step b) which elicit neurite outgrowth from the cultured neurons of step a).

36. An assay method useful to identify arretin antagonist agents that suppress inhibition of neuron growth, comprising the steps of:

a) culturing cells that extend cytoplasmic processes whose growth is inhibited by arretin, on a growth substrate that incorporates a growth-inhibiting amount of arretin; and

b) exposing the cells to a candidate arretin antagonist agent in an amount and for a period sufficient prospectively to permit growth of said cells;

thereby identifying as arretin antagonists said candidates of step b) which elicit changes in cell attachment, cell spreading, cell migration, cell invasiveness or cell morphology from said cultured cells of step a)

37. A method for screening for compounds that stimulate neurite growth, comprising the steps of:

a) coating a growth permissive substrate with a growth-inhibiting amount of arretin; and

b) adding a test compound and arretin-growth-sensitive cells to the arretin-coated substrate;

c) washing to remove unattached cells;

d) measuring the viable cells attached to the substrate,

thereby identifying the cell adhesion candidates of step b) which elicit changes in cell attachment within the cultured cells of step a).

38. A method for inhibiting neuron growth, comprising the step of introducing into the neuron growth environment a growth-inhibiting amount of a neuron growth inhibitor selected from arretin and an arretin agonist.

39. A method according to claim 38, wherein said inhibitor is arretin.

40. A method according to claim 39, wherein said inhibitor is an arretin agonist having arretin-biological activity of inhibiting neurite outgrowth from neurons cultured on a permissive substrate, and is selected from an arretin fragment, an analog of arretin or of the arretin fragment, a derivative of either arretin, the arretin fragment or said analog, an anti-idiotypic arretin antibody or a binding fragment thereof, and a pharmaceutical agent.

41. A method according to claim 40, wherein said arretin agonist is the arretin ectodomain.

42. A method according to claims 38, 39, 40 or 41, wherein said inhibitor is delivered to a patient afflicted with a medical condition selected from epilepsy, neuroblastoma and neuromas.

43. An antisense oligonucleotide having a sequence capable of binding specifically with any sequences of an mRNA molecule which encodes arretin so as to prevent translation of the mRNA molecule.

44. An antisense oligonucleotide having a sequence of binding specifically with any sequences of a cDNA molecule coding for arretin.

45. An antisense oligonucleotide of claim 43 comprising chemical analogues of nucleotides.

46. A pharmaceutical composition comprising an amount of the oligonucleotide of claim 43 effective to reduce expression of arretin by passing through a cell membrane and binding specifically with mRNA encoding arretin in the cell so as to prevent its translation and a pharmaceutically acceptable hydrophobic

carrier capable of passing through a cell membrane.

47. A pharmaceutical composition of claim 46, wherein the oligonucleotide is coupled to a substance which inactivates mRNA.

Figure 1A

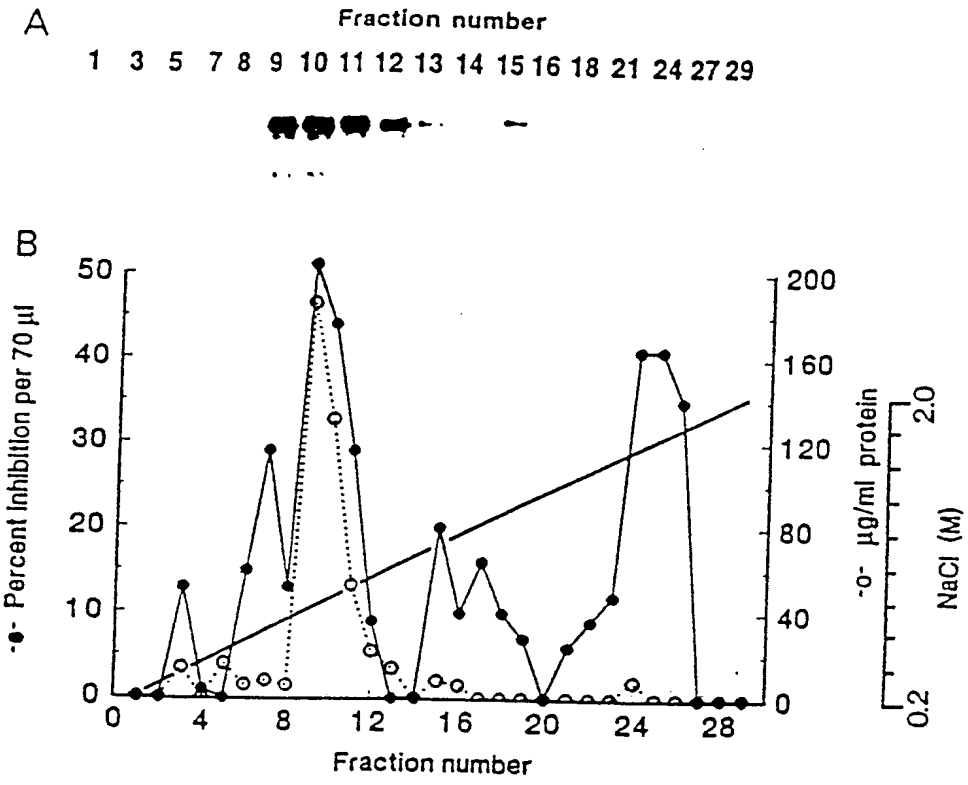


Figure 1B

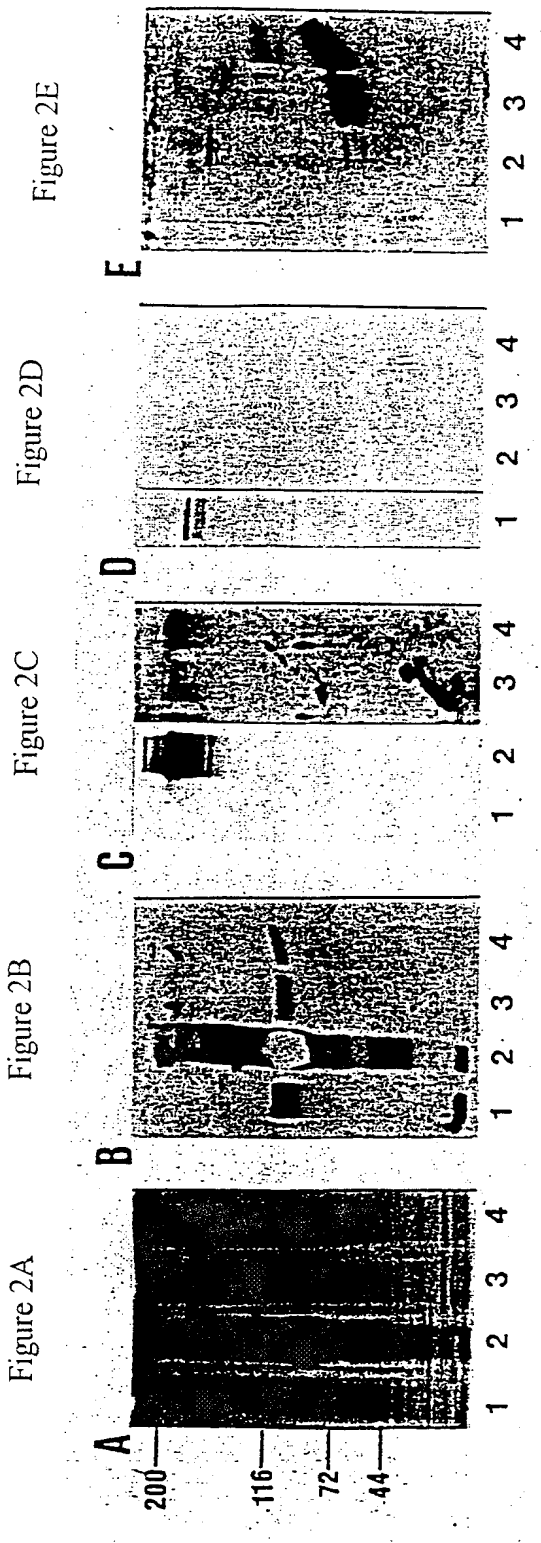


Figure 2A
Figure 2B
Figure 2C
Figure 2D
Figure 2E
A
B
C
D
E
200
116
72
44
1 2 3 4

Figure 3A

Figure 3B

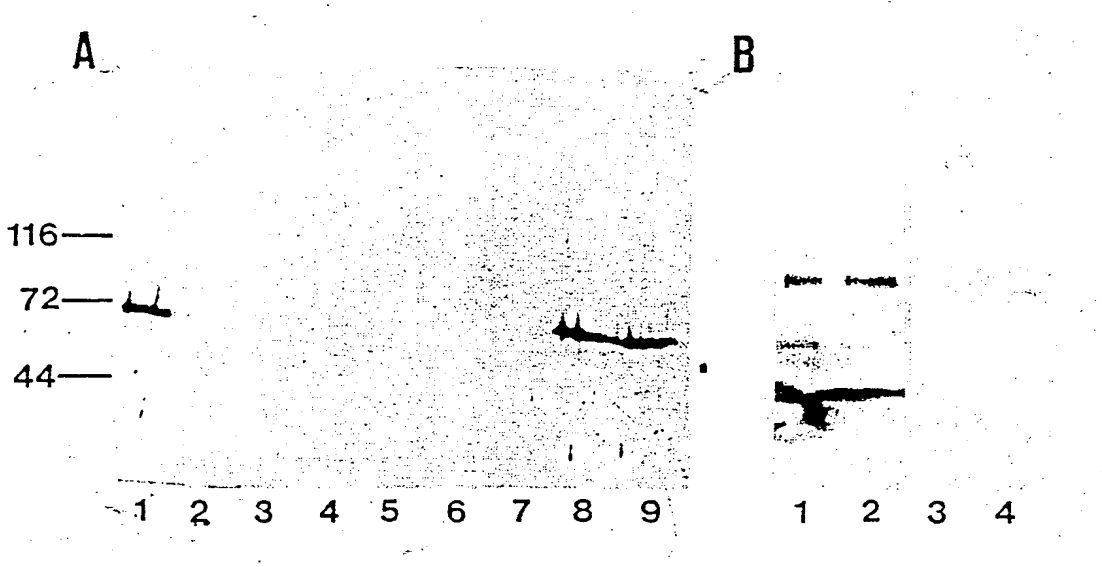


Figure 4A

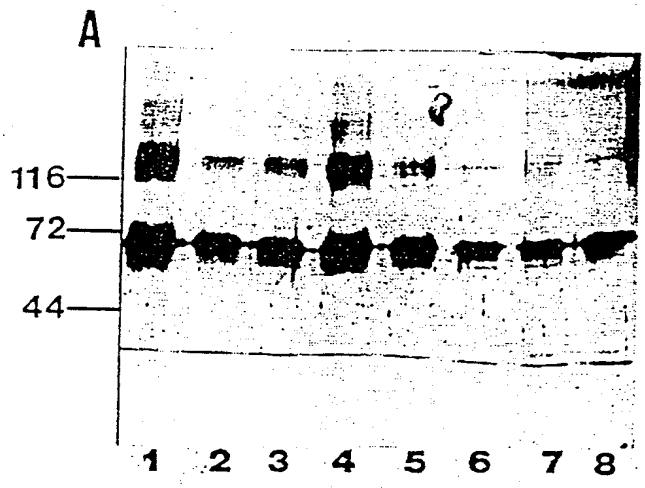
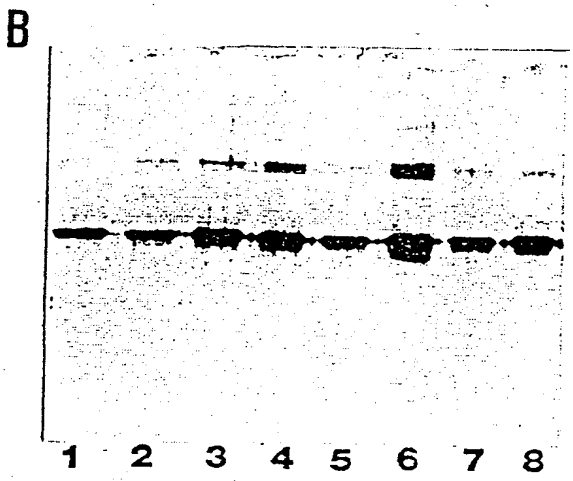


Figure 4B



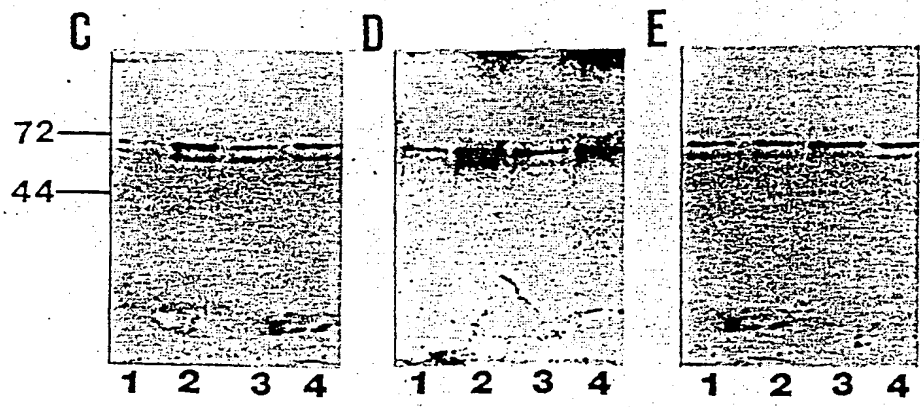


Figure 4C

Figure 4D

Figure 4E

Figure 5A

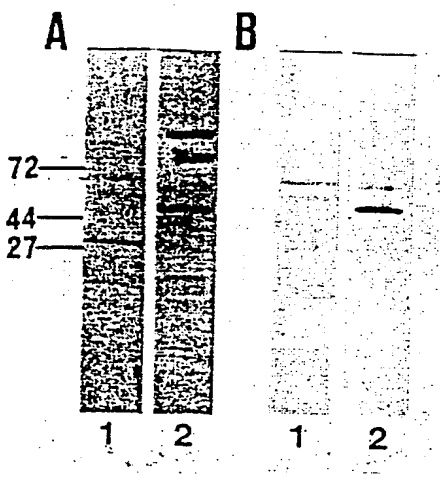


Figure 5B

Figure 6

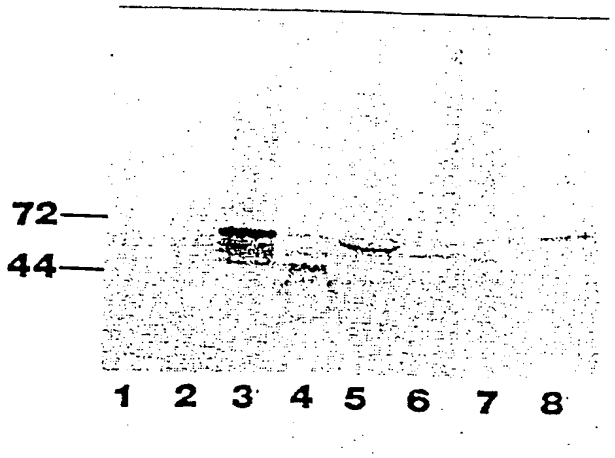


Figure 7

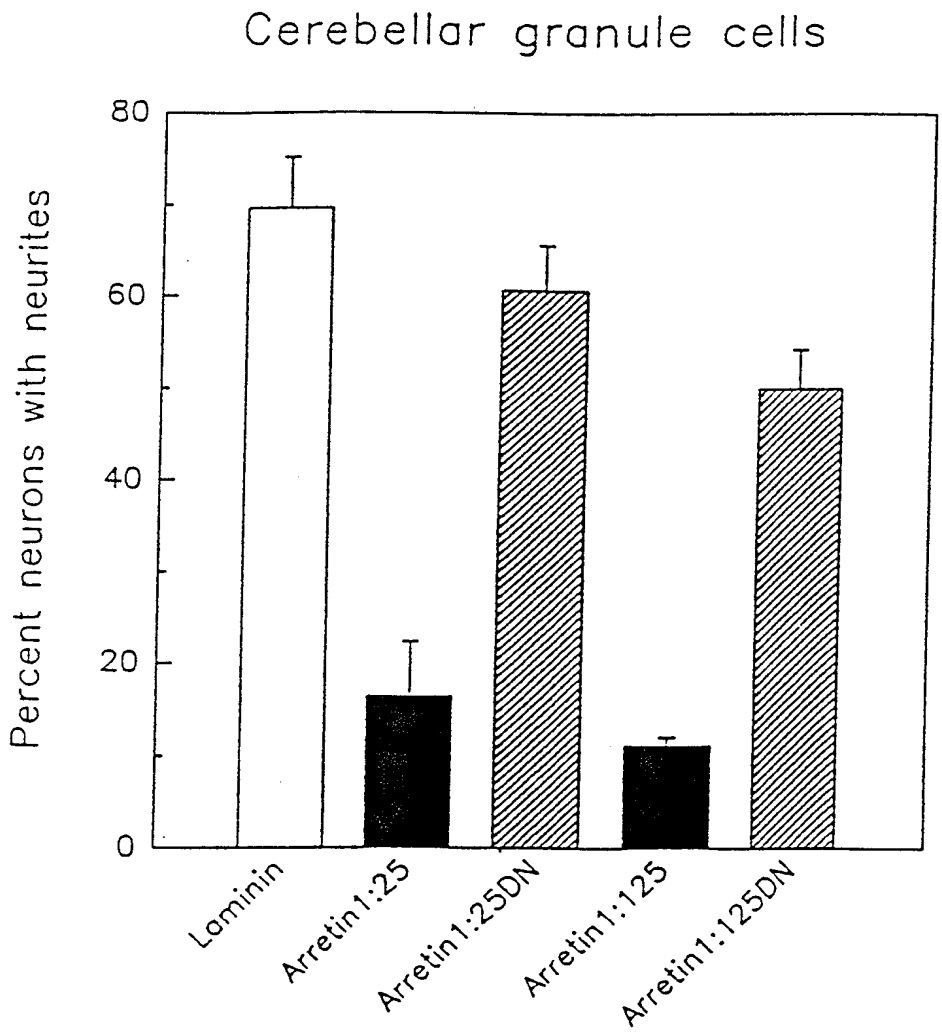

Cerebellar granule cells
Percent neurons with neurites
80
60
40
20
0
Laminin
Arretin1:25
Arretin1:25DN
Arretin1:125
Arretin1:125DN

Figure 8

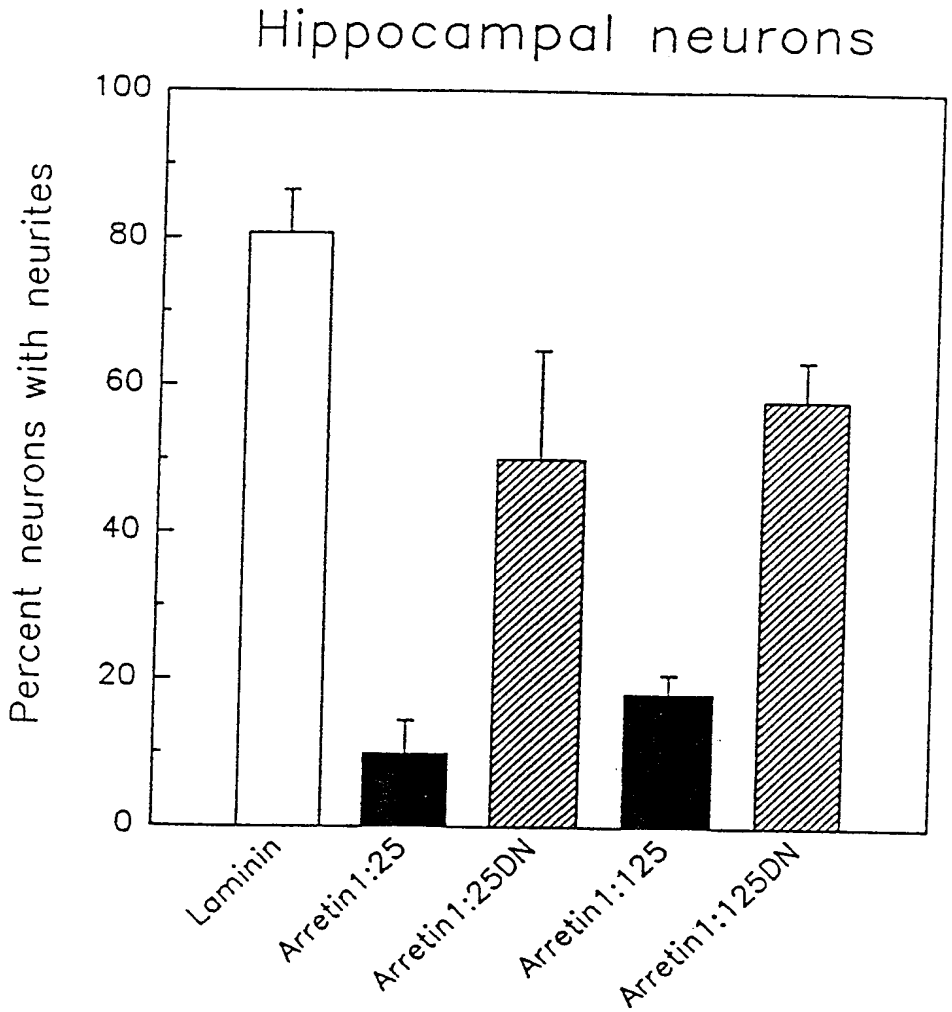

Hippocampal neurons
Percent neurons with neurites
100
80
60
40
20
0
Laminin
Arretin1:25
Arretin1:25DN
Arretin1:125
Arretin1:125DN

Figure 9

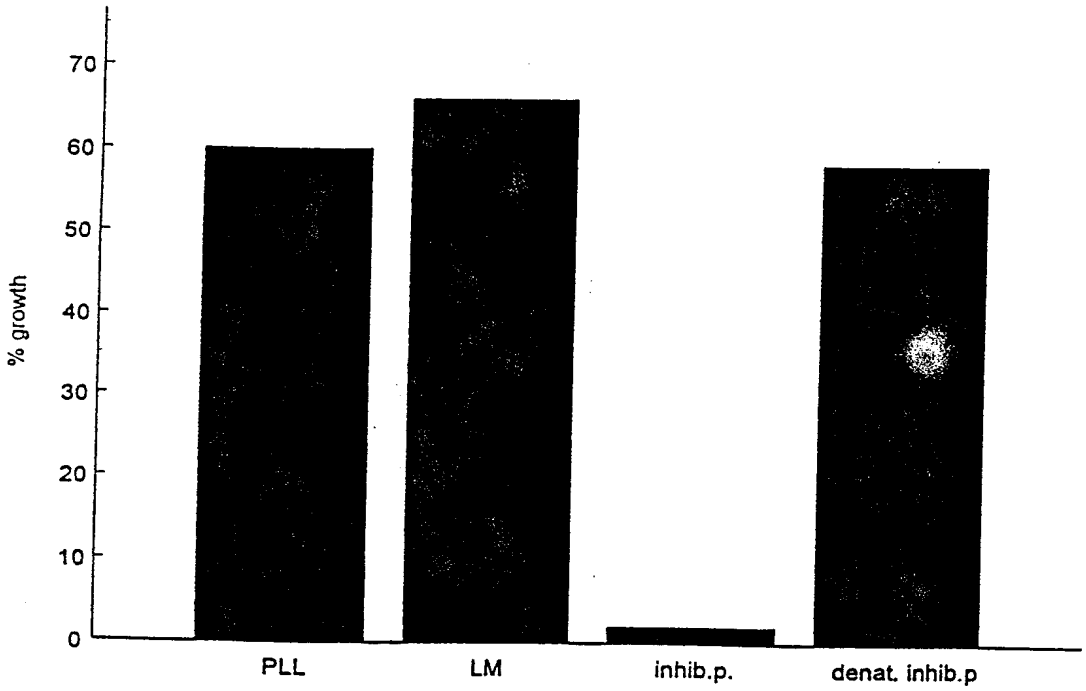


Figure 10

A

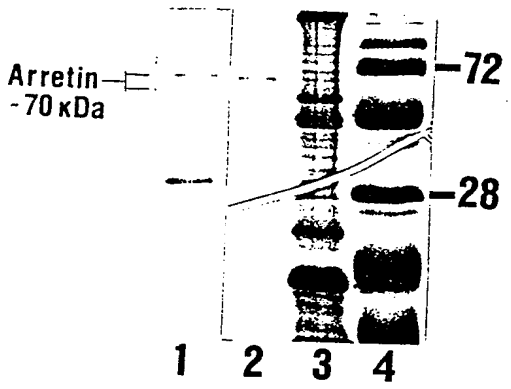


Figure 11

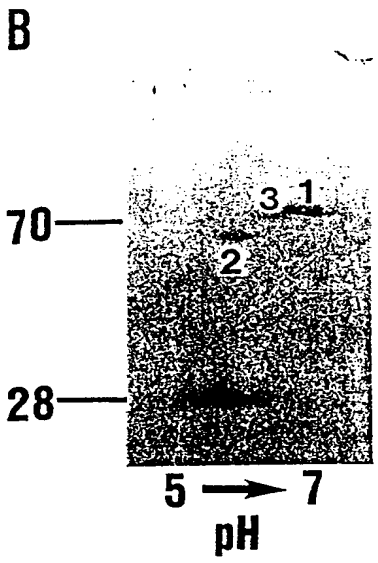


Figure 12A

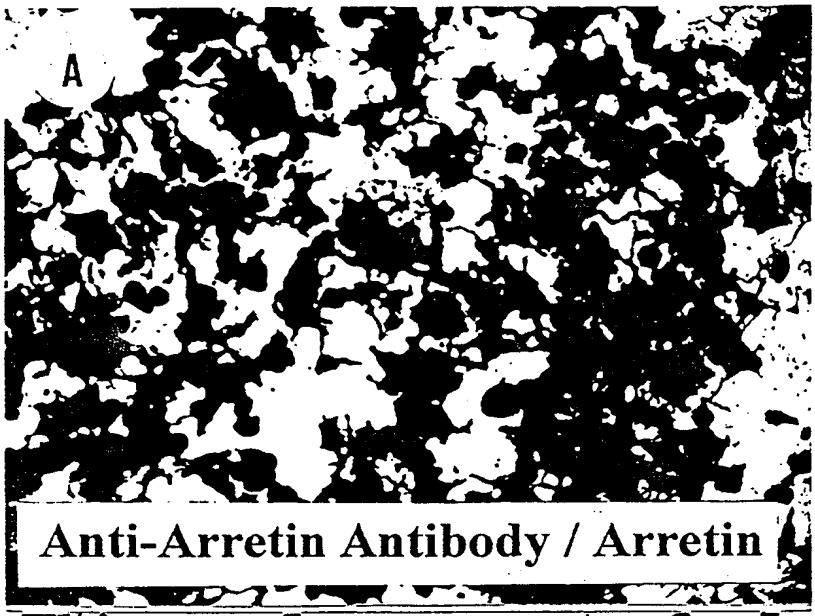


Figure 12B

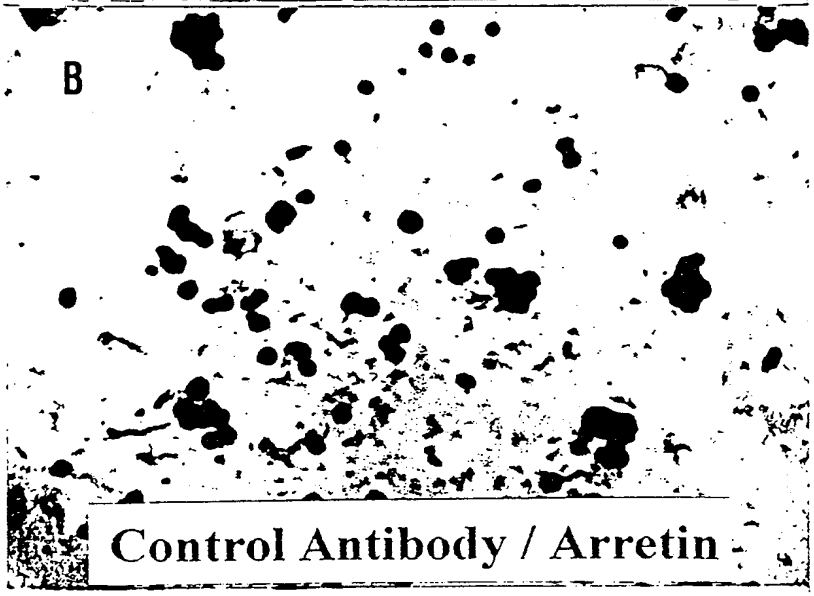


Figure 13

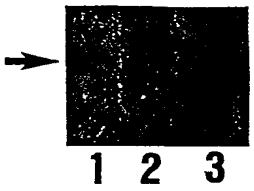


Figure 14A

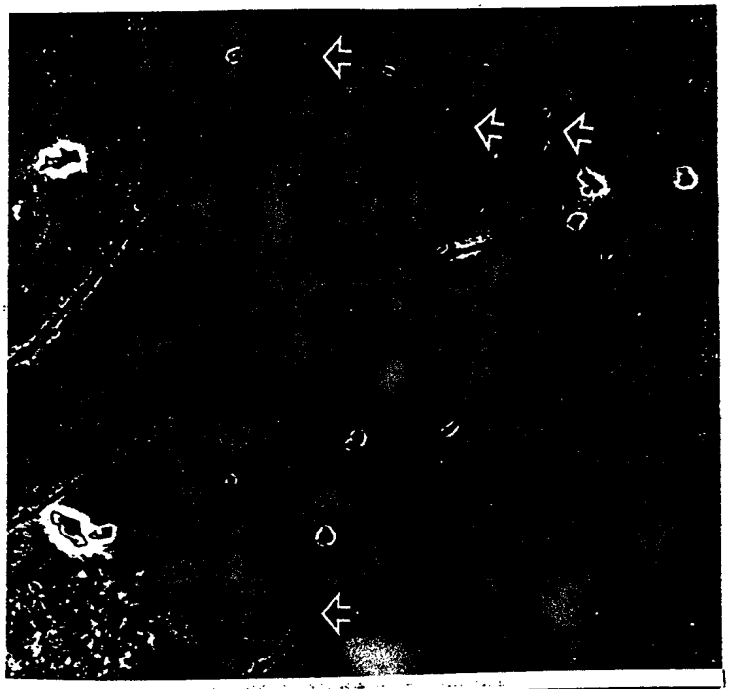
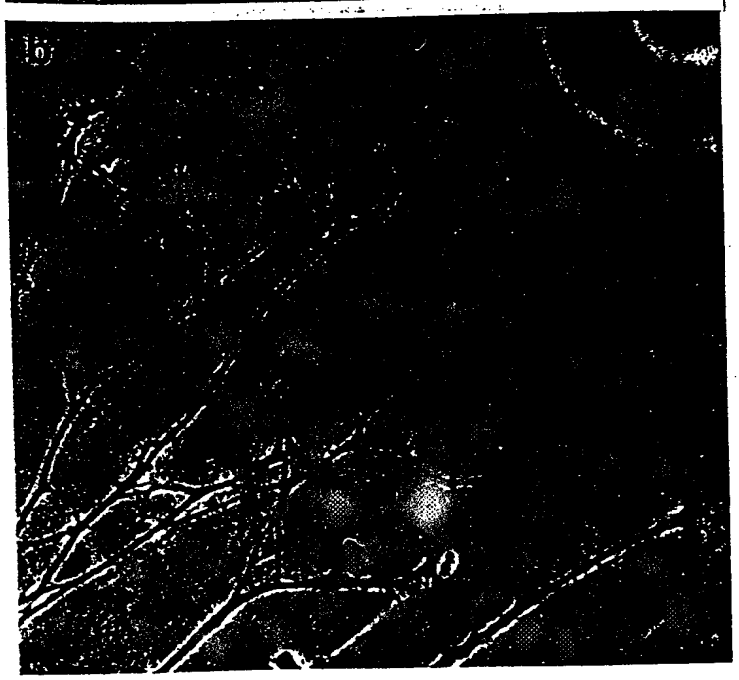


Figure 14B



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(51) International Patent Classification 6 : **C12N 15/11, C07K 14/47, 16/22, A61K 31/70, 39/395, 38/17, G01N 33/68**	**A3**	**(11) International Publication Number:** **WO 98/22499** **(43) International Publication Date:** 28 May 1998 (28.05.98)

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(30) Priority Data:
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(71)(72) Applicants and Inventors: McKERRACHER, Lisa, Joan [CA/CA]; 3816 Draper Avenue, Montreal, Quebec H4A 2D1 (CA). DAVID, Samuel [CA/CA]; 58 Oxford, Baie d'Urfé, Quebec H9X 2T5 (CA). BRAUN, Peter, Erich [CA/CA]; 4098 Highland Avenue, Montreal, Quebec H3G 1Y6 (CA). XIAO, Zhi-Cheng [CN/CN]; Apartment 505, 4530 Cote des Neiges, Montreal, Quebec H3V 1G1 (CA).

(74) Agent: MBM & CO.; P.O. Box 809, Station B, Ottawa, Ontario K1P 5P9 (CA).

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(57) Abstract

The present invention relates to a neuron and neural tumor growth regulatory system, based on the novel protein, arretin and its isoforms and fragments thereof, its receptor, antibodies directed against the components of this system and diagnostic, therapeutic, and research uses for each of these aspects. This protein has an apparent molecular weight of approximately 70 kDa. Embodiments of the invention comprise the amino acid sequence and probes designed therefrom for nucleic acid sequences encoding arretin. Alternatively, tagged arretin protein for use as a reporter to detect receptors of arretin, which are then sequenced and used to obtain probes for the nucleic acid sequences encoding arretin receptors, are included. The present invenetion further relates to arretin receptors and fragments thereof as well as the nucleic acid sequences coding for such arretin receptors and fragments, and their therapeutic and diagnostic uses. Substances which function as either aganists or antagonists to arretin receptors are also envisioned and included within the scope of the present invention.

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INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/CA 97/00868

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/11 C07K14/47 C07K16/22 A61K31/70 A61K39/395
A61K38/17 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 95 22344 A (UNIV MCGILL ;MCKERRACHER LISA JOAN (CA); DAVID SAMUEL (CA); BRAUN) 24 August 1995 see the whole document ---	1-47
A	FRIEDLANDER ET AL.: "THE NEURONAL CHONDROITIN SULFATE PROTEOGLYCAN NEUROCAN BINDS TO THE NEURAL CELL ADHESION MOLECULES Ng-CAM/L1/NILE AND N-CAM, AND INHIBITS NEURONAL ADHESION AND NEURITE OUTGROWTH" THE JOURNAL OF CELL BIOLOGY, vol. 125, no. 3, 1994, pages 669-680, XP002062504 see page 670 left-hand column, paragraph 1 and see page 677, left-hand column, paragraph 1 --- -/--	1-47

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
20 April 1998	20. 05. 1998
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Hagenmaier, S

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INTERNATIONAL SEARCH REPORT

In ıtional Application No
PCT/CA 97/00868

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BODE-LESNIEWSKA ET AL.: "Distribution of the large aggregating proteoglycan versican in adult human tissues" THE JOURNAL OF HISTOCHEMISTRY AND CYTOCHEMISTRY, vol. 44, no. 4, - April 1996 pages 303-312, XP002062505 cited in the application see the whole document	1-47
A	WO 95 20397 A (UNIV NEW YORK) 3 August 1995 see the whole document	1-47
A	WO 94 03601 A (UNIV NEW YORK) 17 February 1994 see the whole document	1-47
A	WO 96 32959 A (ACORDA THERAPEUTICS) 24 October 1996 see the whole document	1-47
A	WO 96 32476 A (MOUNT SINAI HOSPITAL CORP ;ROACH ARTHUR (CA); LOZANO ANDRES (CA);) 17 October 1996 see the whole document	1-47
A	WO 95 26201 A (JOLLA CANCER RES FOUND) 5 October 1995 see the whole document	1-47
A	WO 94 17831 A (ZUERICH ERZIEHUNGSDIREKTION) 18 August 1994 see the whole document	1-47
A	SCHWAB M E ET AL: "INHIBITORS OF NEURITE GROWTH" ANNUAL REVIEW OF NEUROSCIENCE, vol. 16, 1 January 1993, pages 565-595, XP000576555 cited in the application see the whole document	1-47
A	ESKO J.: "GENETIC ANALYSIS OF PROTEOGLYCAN STRUCTURE, FUNCTION AND METABOLISM" CURRENT OPINION IN CELL BIOLOGY, vol. 3, 1991, pages 805-816, XP002062506 see the whole document	1-47

2

INTERNATIONAL SEARCH REPORT

In ational application No.
PCT/CA 97/ 00868

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 27-32,42(completely), 38-41 (partially, as far as an in vivo method is concerned) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. ional Application No
PCT/CA 97/00868

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9522344 A	24-08-95	AU 1703595 A	04-09-95
		CA 2183630 A	24-08-95
WO 9520397 A	03-08-95	US 5625040 A	29-04-97
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		CA 2141600 A	17-02-94
		US 5648465 A	15-07-97
WO 9632959 A	24-10-96	AU 5557296 A	07-11-96
		EP 0821591 A	04-02-98
		NO 974811 A	19-12-97
WO 9632476 A	17-10-96	AU 5264496 A	30-10-96
		CA 2174025 A	14-10-96
		CA 2217731 A	17-10-97
		EP 0821731 A	04-02-98
WO 9526201 A	05-10-95	NONE	
WO 9417831 A	18-08-94	AU 5891394 A	29-08-94
		EP 0634939 A	25-01-95
		JP 7509002 T	05-10-95
		ZA 9400887 A	23-08-94

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NEURON AND NEURAL TUMOR GROWTH REGULATORY SYSTEM, ANTIBODIES THERETO AND USES THEREOF

BACKGROUND

Following trauma in the adult central nervous system (CNS) of mammals, injured neurons do not regenerate their transected axons. An important barrier to regeneration is the axon growth inhibitory activity that is present in CNS myelin and that is also associated with the plasma membrane of oligodendrocytes, the cells that synthesize myelin in the CNS (see Schwab, *et al.*, Ann. Rev. Neurosci., 16, 565-595, 1993 for review). The growth inhibitory properties of CNS myelin have been demonstrated in a number of different laboratories by a wide variety of techniques, including plating neurons on myelin substrates or cryostat sections of white matter, and observations of axon contact with mature oligodendrocytes (Schwab *et al.*, 1993). Therefore, it is well documented that adult neurons cannot extend neurites over CNS myelin *in vitro*.

It has also been well documented that removing myelin *in vivo* improves the success of regenerative growth over the native terrain of the CNS. Regeneration occurs after irradiation of newborn rats, a procedure that kills oligodendrocytes and prevents the appearance of myelin proteins (Savio and Schwab, Neurobiology, 87, 4130-4133, 1990). After such a procedure in rats and combined with a corticospinal trait lesion, some corticospinal axons regrow long distances beyond the lesions. Also, in a chick model of spinal cord repair, the onset of myelination correlates with a loss of its regenerative ability of cut axons (Keirstead, *et al.*, Proc. Nat. Acad. Sci. (USA), 89, 11664-11668, 1992). The removal of myelin with anti-galactocerebroside and complement in the embryonic chick spinal cord extends the permissive period for axonal regeneration. These experiments demonstrate a good correlation between myelination and the failure of axons to regenerate in the CNS.

Until recently the identity of specific proteins important for the inhibitory activity remained elusive, although they have been sought since 1988 (Schwab *et al.*, 1993). One component of the myelin-derived inhibitors as myelin-associated glycoprotein (MAG) has been identified (McKerracher *et al.*, Neuron, 13, 229-246 and 805-811, 1994). This finding was at first surprising because MAG does not have the biochemical properties or distribution of the myelin-derived inhibitor reported by Schwab *et al.*, (1993).

There have been some expectations of the properties of the non-MAG inhibitor in myelin, based on the work of Martin Schwab (reviewed in detail by Schwab *et al.*, 1993). It was reported to be attributed to two different proteins of 35 kDa and 250 KDa. Myelin- derived growth inhibitory activity was also reported to be a property of CNS myelin but not PNS myelin. It has since been determined that PNS has inhibitory activity, but the inhibitory activity is masked by laminin (David *et al.*, 42, 594-602, 1995).

Schwab has sought to determine the identity of the myelin-derived inhibitors of neurite outgrowth, and his findings have been extensively reviewed (Schwab *et al.*, 1993). Schwab determined a possible molecular weight of the growth inhibitory proteins in the following way. Myelin proteins were separated by SDS PAGE under denaturing conditions, the gel was cut into slices and proteins were eluted from the slices and inserted into liposomes. The liposomes were tested for inhibitory activity. Regions of the gel corresponding to 250 kDa and 35 kDa were identified as most inhibitory, and heat destroyed the inhibitory activity. The loss of activity with heat suggested that the activity was due to a protein that required native conformation. Why this putative protein retains biological activity after the denaturing conditions of SDS-PAGE remain a mystery. The evidence to claim the 250 kDa and 35 kDa proteins as the major myelin inhibitors is weak.

The evidence for the 250 kDa and 35 kDa proteins as myelin-derived inhibitors comes

mainly from the work of Schwab with their IN-1 antibody. Schwab raised monoclonal antibodies to the inhibitory proteins eluted from gels and cloned one monoclonal antibody, called IN-1, which is a low-affinity IgM. It has been used to characterize the myelin-derived inhibition. The antibody is reported to bind to the 35 kDa and 250 kDa proteins, but the Western blots indicate that it lacks specificity and that many additional bands are also recognized (Caroni and Schwab, Neuron, 1, 85-96, 1988). The immunoprecipitation data presented in the same publication was given in tabular form rather than by showing the gels, as a rigorous analysis requires, and these data cannot be easily evaluated. However, application of the antibody to various *in vitro* preparations has been shown to partially block the inhibitory properties of myelin. Also, the application of this antibody *in vivo* allows a small number of corticospinal axons to elongate long distances after CNS injury (Schnell and Schwab, Nature, 343, 269-272, 1990; Schnell *et al.*, Nature, 367, 170-173, 1994). Moreover, raphe spinal serotonergic neurons also regenerate, and there is improvement in some aspects of locomotor function (Bregman *et al.*, Nature, 378, 498, 1995). Therefore, the evidence to date suggests that blocking the myelin-derived inhibitors of neurite outgrowth will be an important component of any therapeutic strategy to improve regeneration in the adult CNS. Because the proteins identified by the antibodies have not been identified, the components of myelin that block axon growth, in addition to MAG, remain unknown. It has been noted that both MAG and the new inhibitor arretin, that is described herein, appear to be acidic proteins. Therefore, to date, the identity of the non-MAG inhibitory components of myelin remain unknown, and the proteins that the IN-1 antibody recognizes remain uncharacterized.

While the findings of MAG as an inhibitor of neurite outgrowth were surprising, other laboratories have now substantiated our *in vitro* documentation that MAG is an important myelin-derived inhibitor of neurite growth (Mukhopadhay *et al.*, Neuron, 13, 757-767, 1994; Schafer *et al.*, Neuron, In press, 1996; DeBellard, Mol. Cell Neurosci., 7, 7616-7628, 1996). The contribution of MAG has also been examined *in vivo*, and the results indicate that other growth inhibitory proteins in myelin exist (Li *et al.*, J.

Neurosci. Res., In press, 1996). In these studies it has been shown that some differences occur in axon extension after lesions in MAG null mutant mice, a finding that differs from that reported for a similar study of a different line of MAG-deficient mice (Bartsch *et al.*, Eur. J. Neurosci., 7, 907-916, 1995; Bartsch *et al.*, Neuron, 15, 1375-1381, 1995). In both cases, however, the results from the studies of MAG knock out mice injured in the CNS are less dramatic than reported with treatment with the IN-1 antibody (Bartsch *et al.*, 1995 - see below), suggesting the non-MAG inhibitors that remain in CNS myelin form an important barrier to regeneration; indeed their expression in the absence of MAG expression may have been upregulated during CNS development.

Data has suggested that MAG may not be acting alone. To date, the presence of another protein had not been shown nor were its properties known. The present invention has, for the first time, demonstrated the presence and properties of such a protein.

Tenascins

Four members of the tenascin family have been identified and characterized:
tenascin-C,
tenascin-R, tenascin-X and tenascin-Y (Bristow *et al.*, Cell Biol., 122, 265-278, 1993; Erickson, H.P., J. Cell Biol., 120, 1079-1081, 1993). Tenascin-X and tenascin-Y are not prominent in the nervous system. Tenascin-C is important in the development of the nervous system and it is the best characterized member of this protein family. It is generated by alternative splicing (Weller *et al.*, J. Cell Biol., 112, 355-362, 1991; Sriramarao and Bourdon, Nucl. Acids Res., 21, 347-362, 1993) and the variants are expressed both in the nervous system and in several non-neural tissues. Tenascin-C has been suggested to be involved in neuron-glia adhesive and migratory events and to promote axon outgrowth after injury of peripheral nerves.

Tenascin-R (TN-R), has a modular structure similar to TN-C, previously designated J1-160/180 and janusin in rodents, or restriction in chicken (Pesheva *et al.*, J. Cell

Biol., 109, 1765-1778, 1989; Fuss *et al.*, J. Neurosci. Res., 29, 299-307, 1991, and J. Cell Biol., 120, 1237-1249, 1993). Tenascin-R is predominantly expressed by oligodendrocytes during the onset and early phases of myelin formation and remains detectable in myelin-forming oligodendrocytes in the adult, and is also expressed by neurons (Pesheva *et al.*, 1989; Fuss *et al.*, 1993). Tenascin-R has been shown to be involved in promotion of neurite outgrowth and morphological polarization of differentiating neurons when presented as a uniform substrate (Lochter and Schachner, J. Neurosci., 13, 3986-4000, 1993; Lochter *et al.*, Eur. J. Neurosci., 6, 597-606, 1994). When offered as a sharp substrate boundary with a neurite outgrowth conducive molecule, tenascin-R is repellent for growth cone advance (Taylor *et al.*, J. Neurosci. Res., 35, 347-362, 1993; Pesheva *et al.*, 1993).

Tenacins are not thought to be an important component of the myelin-derived inhibitory activity because they lack the specific myelin distribution, they are not restricted to the CNS, and their molecular weight differs from the presumptive proteins identified by Schwab. However, studies have indicated that both tenascin R and tenascin C are minor inhibitory components of octlyglucoside extracts of myelin. The data indicate that growth inhibitory proteins from the CNS matrix may become associated with isolated myelin fragments.

Chondroitin Sulfate Proteoglycans (CSPGs)

Proteoglycans (PGs) are proteins that are found predominantly on the cell surface and in the extracellular matrix; they are covalently bound to complex carbohydrates called glycosaminoglycans. Glycosaminoglycans (GAGs) are polymers of disaccharide repeats, which are mostly highly sulphated and negatively charged. The main glycosaminoglycans in PGs are chondroitin sulfate, dermatan sulfate, heparan sulfate, and keratan sulfate. (Ruoslahti, E., Ann. Rev. Cell Biol., 4, 229-255, 1988). The number of GAG chains can vary from one to over one hundred.

Proteoglycans are known to be important for the development and regeneration of the

nervous system, but they have not been considered to be myelin proteins or form part of the growth inhibitory activity of myelin. Moreover, proteoglycans have not been reported to be recognized by the IN-1 antibody or to form a major growth inhibitory component of white matter regions of the CNS.

Chondroitin sulfate proteoglycans (CSPGs) constitute the major population of PGs in the CNS. The different patterns of localization and developmental expression of CSPGs throughout the nervous system implicate them in diverse roles in development and in regeneration. After injuries in the adult CNS, CSPGs are thought to be important in the formation of the glial scar. They have been implicated as both positive and negative modulators of axonal growth. Recent observations indicate that DSD-1-PG, a neural chondroitin sulfate proteoglycan, promotes neurite outgrowth of embryonic day 14 mesencephalic and embryonic day 18 hippocampal neurons from rat (Faissner *et al.*, J. Neurochem., 54, 1004-1015, 1994). However, NG2, an integral membrane CSPGs expressed on the surface of glial progenitor cells, inhibits neurite growth. The NG2 proteoglycan also inhibits neurite growth after digestion with chondroitinase ABC, indicating that the inhibitory activity is a property of the core protein and not the covalently attached chondroitin sulfate glycosaminoglycan chains (Dou and Levine, J. Neurosci., 14, 7616-7628, 1994), but for many other types of CSPGs the inhibitory activity resides in the glycosaminoglycan. Chondroitin sulfate proteoglycan immunoreactivity is increased after cerebral cortical (McKeon *et al.*, J. Neurosci., 11, 3398-3411, 1991), spinal (Pindzola *et al.*, Dev. Biol., 156, 34-48, 1993) and optic nerve lesions (Brittis *et al.*, Science, 255, 733-736, 1992). *In vitro* studies indicate that CSPG immunoreactivity on astrocytes increases when they are plated on monolayers of leptomeningeal cells (Ness and David, Glia, In press, 1997). Similar increases in CSPG immunoreactivity have been reported on Schwann cells co-cultured with astrocytes (Ghimikar and Eng, Glia, 14, 145-152, 1995). This highly sulfated proteoglycan which is a potent inhibitor of neurite growth in vitro (Snow *et al.*, Neurol, 109, 111-130, 1990), has been shown to be involved in the differentiation of developing retinal ganglion cells, and by acting as an inhibitory substrate serves to appropriately

guide ganglion cell axons toward the optic disc (Brittis and Silver, Proc. Nat. Acad. Sci. USA., 19, 7539-7542, 1992). McKeon *et al.,* J. Neurosci., 11, 3398-3411, 1991) have reported that astrocytes harvested from the site of cerebral cortical lesions express increased amounts of CSPG, which reduces neurite growth on these cells in vitro. The expression of CSPG on the surface of a subset of cultured astrocytes has also been shown to correlate with their reduced capacity to support neurite growth (Meiners *et al.*, J. Neurosci., 15, 8096-8108, 1995). The collapse of the growth cone is an important response of the growing exon to inhibitory cues in the environment. Collapse of the lamellipodium is sometimes followed by retraction of the neurite (Kapfhammer and Raper, J. Neurosci., 7, 201-212, 1987; Raper and Grunewald, Exp. Neurol., 109, 70-74, 1990; Bandtlow *et al.,* J. Neurosci., 10, 3837-3848, 1990). Many previously characterized inhibitory molecules found in the developing nervous system have been shown to cause growth cone collapse in vitro (Davies *et al.*, Neuron, 4, 11-20, 1990; Stahl *et al.*, Neuron, 5, 735-743, 1990; Bandtlow *et al.*, 1990; Keynes *et al.*, Ann. N.Y. Acad. Sci. 633, 562, 1991; Luo *et al.*, Cell, 75, 217-227, 1993). Such collapsing activity has been observed previously in the adult chicken brain and shown to bind to PNA, and be associated with glycoproteins with molecular weights of 48 and 55 kDa (Keynes *et al.*, 1991). Others, such as the 33 kDa inhibitor in the developing chicken tectum also binds to PNA (Stahl *et al*, 1990). Because proteoglycans are a very heterogenous class of proteins with diverse biological activities it is essential that individual, identified proteins be considered. Relevant to the present invention are the proteoglycans phosphocan and versican, because the protein of the present invention, arretin, has common immunological eptitopes with these proteins.

Phosphacan.

Phosphacan is a proteoglycan in brain recognized by the 3F8 antibody (Maurel *et al.*, Proc. Nat. Acad. Sci. USA, 91, 2512-2516, 1994), and by the 6B4 antibody (Maeda *et al.*, Neurosci., 67, 23-35, 1995). Phosphacan is a splice variant of a receptor-type protein tyrosine phosphatase, although phosphacan itself lacks the phosphatase domains. It is a protein with an apparent molecular weight of approximately 500 kDa,

having a core glycoprotein of approximately 400 kDa. The HNK-1 monoclonal antibody recognizes a 3-sulphated carbohydrate epitope, and this epitope is strongly represented in phosphacan from 7-day brain, but not in adult brain (Rauch *et al.*, J. Biol. Chem., 266, 14785-14801, 1991). In development phosphacan is immunostained on radial glia and on neurons (Maeda *et al.*, 1995) and generally it is expressed in both white matter and grey matter regions (Meyer-Puttlitz, *et al.*, J. Comp. Neurol. 366, 44-54, 1996). and therefore, unlike the myelin inhibitors, it is not localized only to white matter areas. It appears to be synthesized only by astroglia (Engel *et al*, 1996).

Versican.

Versican, a CSPG originally isolated from fibroblasts, also called PG-M, has an apparent molecular weight of approximately 900 kDa, with a core protein of approximately300 to 400 kDa (Braunewell *et al.*, Eur. J. Neurosci., 7, 792-804, 1995; Naso *et al*, 1994). Versican belongs to a family of aggregating CSPGs; other members of the family include the cartilage-derived aggrecan, and two PGs expressed in the nervous system, neurocan and brevican (Dours-Zimmermann and Zimmerermann, J. Biol. Chem., 269, 32992-32998, 1994). Versican is widely distributed in adult human tissues, associated with connective tissue of various organs, in certain muscle tissues, epithelia, and in central and peripheral nervous tissues. Four versican isoforms are known (Vo, V1, V2, V3), derived by alternative splicing. They vary in calculated mass from approximately 370 kDa (Vo) to approximately 72 kDa (V3). It has been suggested that the association of versican expression with cell migration and proliferation *in vivo* and its adhesion inhibitory properties in vitro point to pathological processes such as tumorigenesis and metastasis (Bode-Lesniewska *et al.*, Histol. & Cyto., 44, 303-312, 1996; Naso *et al,*. J. Biol. Chem., 269, 32999-33008, 1994).

Other CSPGs related to versican are brevican (Mr approximately 145 kDa) and neurocan (Mr > 300 kDa). Neither of these is known to be expressed by oligodendrocytes and are therefore not expected to be present in CNS myelin (Engel *et al.*, J. Comp. Neurol. 366, 34-43, 1996; Yamada *et al.*, J. Biol. Chem., 269, 10119-

10126, 1994).

Another CSPG family member that is not related to either versican or phosphacan, is NG2. Although it is expressed by O2A progenitor cells in the developing rat nervous system, it has no apparent homology to arretin-relevant GSPG's, and has an Mr approximately 400-800 kDa with a core protein of approximately 300 kDa (Nishiyama *et al.*, J. Cell Biol., 114, 359-371, 1991).

Neuroblastoma

Neuroblastoma arises from neuroectoderm and contains anaplastic sympathetic ganglion cells (reviewed in Pinkel and Howarth, 1985, In: Medical Oncology, Calabrese, P., Rosenberg, S. A., and Schein, P. S., eds., MacMillan, N.Y., pp. 1226-1257). One interesting aspect of neuroblastoma is that it has one of the highest rates of spontaneous regression among human tumors (Everson, 1964, Ann. N.Y. Acad. Sci. 114:721-735) and a correlation exists between such regression and maturation of benign ganglioneuroma (Bolande, 1977, Am. J. Dis. Child. 122:12-14). Neuroblastoma cells have been found to retain the capacity for morphological maturation in culture. The tumors may occur anywhere along the sympathetic chain, with 50% of such tumors originating in the adrenal medulla.

Neuroblastoma affects predominantly preschool aged children and is the most common extracranial solid tumor in childhood, constituting 6.5% of pediatric neoplasms. One half are less than two years of age upon diagnosis. Metastases are evident in 60% of the patients at presentation usually involving the bones, bone marrow, liver, or skin. The presenting symptoms may be related to the primary tumor (spinal coral compression, abdominal mass), metastatic tumor (bone pain) or metabolic effects of substances such as catecholamines or vasoactive polypeptides secreted by the tumor (e.g. hypertension, diarrhea). Experimental evidence indicates that an altered response to NGF is associated with neuroblastoma (Sonnenfeld and Ishii, 1982, J. Neurosci. Res.

8:375-391). NGF stimulated neurite outgrowth in one-half of the neuroblastoma cell lines tested; the other half was insensitive. However, NGF neither reduced the growth rate nor enhanced survival in any neuroblastoma cell line.Present therapies for neuroblastoma involve surgery and/or chemotherapy. Radiation therapy is used for incomplete tumor responses to chemotherapy. There is a 70-100% survival rate in individuals with localized tumors, but only a 20% survival rate in those with metastatic disease even with multiagent chemotherapy. It appears that patients less than one year have a better prognosis (70%) than older children.

This background information is provided for the purpose of making known information believed by the applicant to be of possible relevance to the present invention. No admission is necessarily intended, nor should be construed, that any of the preceding information constitutes prior art against the present invention. Publications referred to throughout the specification are hereby incorporated by reference in their entireties in this application.

SUMMARY OF THE INVENTION

The present invention relates to a neuron and neural tumor growth regulatory system, antibodies directed against the components of this system and diagnostic, therapeutic, and research uses for each of these aspects. The concept of a system is used to denote the functional relationship between the genes (for the regulatory factors and the receptors), their encoded protein-regulatory factors which regulate neuron growth (particularly neurite growth), and the receptors which are activated by the protein. The functional relationship allows one to use one component to identify and determine another. For example, having identified the protein component (factor or receptor), one can use techniques well known in the art to identify the gene.

In accordance with the present invention, a protein has now been identified, hereinafter

referred to as arretin, as one of the molecular components involved in contact-mediated growth inhibition on myelin. This protein has an apparent molecular weight of approximately 70 kDa, but it could be derived from a molecular complex. Given the purified protein, procedures for obtaining the other parts of the system are well known to those skilled in the art to purify the other components to the system. For example, the protein can be used in very standard techniques to obtain the amino acid sequence which can be used to obtain probes for nucleic acid sequences encoding arretin. Alternatively, arretin protein may be tagged for use as a reporter to detect receptors of arretin, which are then sequenced and used to obtain probes for the nucleic acid sequences encoding arretin receptors. Moreover, the production of antibodies to each of these components is also standard procedure.

The present invention further relates to arretin receptors and fragments thereof as well as the nucleic acid sequences coding for such arretin receptors and fragments, and their therapeutic and diagnostic uses. Substances which function as either agonists or antagonists to arretin receptors are also envisioned and within the scope of the present invention.

The present invention further relates to the nucleic acid sequences coding for arretin and its receptors, in addition to their therapeutic and diagnostic uses.

In accordance with another aspect of the present invention, there is provided the use of arretin for the regulation of growth of neurons and neural tumors.

In a further aspect of the present invention, there is provided a method for inhibiting growth of neural tumors, comprising the steps of introducing into the growth environment of the neurons a growth inhibiting amount of arretin, fragments thereof, or an arretin agonist.

In yet a further aspect of the present invention, arretin can be used to design small

molecules to block neurite outgrowth and neural tumor growth. These small molecules will be useful to block growth in situations involving aberrant sprouting, epilepsy, or metastasis.

A further embodiment involves a method of suppressing the inhibition of neuron growth, comprising the steps of delivering to the nerve growth environment, antibodies directed against arretin in an amount effective to reverse said inhibition.

In another aspect of the present invention arretin can be used to design antagonist agents that suppress the arretin-neuronal growth regulatory system. These antagonist agents can be used to promote axon regrowth and recovery from trauma or neurodegenerative disease.

In accordance with another aspect of the present invention, there is provided an assay method useful to identify arretin antagonist agents that suppress inhibition of neuron growth, comprising the steps of:

a) culturing neurons on a growth permissive substrate that incorporates a growth-inhibiting amount of arretin; and

b) exposing the cultured neurons of step a) to a candidate arretin antagonist agent in an amount and for a period sufficient prospectively to permit growth of the neurons;

thereby identifying as arretin antagonists the candidates of step b) which elicit neurite outgrowth from the cultured neurons of step a).

In yet another aspect of the present invention, there is provided an assay method useful for screening for compounds that stimulate cell adhesion and neurite growth, comprising the steps of:

a) coating a growth permissive substrate with a growth-inhibiting amount of arretin; and

b) adding a test compound and neuronal cells to the arretin-coated substrate;

c) washing to remove unattached cells;

d) measuring the viable cells attached to the substrate,

thereby identifying the cell adhesion candidates of step b) which elicit neurite outgrowth from the cultured neurons of step a).

In accordance with another aspect of the present invention, there is provided a method to suppress the inhibition of neurons, comprising the steps of delivering, to the nerve growth environment, an antagonist for arretin or its receptor in an amount effective to reverse said inhibition.

In another embodiment, the nucleic acids encoding arretin and/or its receptor can be used in antisense techniques and therapies.

Arretin inhibits neurite outgrowth in nerve cells and neuroblastoma cells. Such inhibitory protein comprises a 70,000 dalton molecular weight protein, aggregates, and analogs, derivatives, and fragments thereof. Arretin and its related proteins proteins may be used in the treatment of patients with malignant tumors which include but are not limited to melanoma and nerve tissue tumors (e.g., glioma, or neuroblastoma). The present invention also relates to antagonists of arretin, including, but not limited to, antibodies. Such antibodies can be used to neutralize the neurite growth inhibitory factors for regenerative repair after trauma, degeneration, or inflammation. In a further specific embodiment, monoclonal antibody may be used to promote regeneration of nerve fibers over long distances following spinal cord damage.

Various other objects and advantages of the present invention will become apparent from the detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Analysis of growth inhibition after separation of myelin proteins by DEAE

anion exchange chromatography.

A. Western blots of column fractions probed with anti-MAG antibody.

B. Neurite growth inhibition and protein profile present in the column fraction shown in A.

Figure 2. Identification of 70 kDa components in DEAE chromatographic fractions from CNS myelin as chondroitin sulphate proteoglycans. Myelin extracts (lane 1), DEAE chromatographic fractions 10, 25, and 32 (lanes 2, 3, and 4) were subjected to SDS-PAGE (6-16% acrylamide gradient) under reducing conditions, and detected by silver staining (A) and Western blots with anti-MAG (B), anti-TN-C (C), anti-TN-R (D), and anti-CS 473 antibodies (E). The position and molecular weight in kDa of marker proteins is indicated.

Figure 3. Western blot analysis of PNA affinity purification of the 70 kDa CSPGs from DEAE chromatographic fractions 20-34. A. Pooled DEAE chromatographic fractions 20-34 (lane 1), fractions 2 and 6 (lanes 2 and 3) of Hepes buffer wash, fractions 2 and 6 (lanes 4 and 5) of high salt buffer wash, and fractions 2, 4, 6, and 8 (lanes 6, 7, 8, and 9) were subjected to SDS-PAGE (6-16% acrylamide gradient) under reducing conditions, and detected by Western blots with anti-CS 473 antibody. B. Pooled DEAE chromatographic fractions 20-34 (lane 1), flow-through of PNA affinity column (lane 2), fraction 2 (lane 3) and pooled eluate (lane 4) were subjected to SDS-PAGE (6-16% acrylamide gradient) under reducing conditions, and detected by Western blots with anti-MAG antibodies. The position and molecular weight in kDa of marker proteins is indicated.

Figure 4. Identification of the 70 kDa components as phosphacan and versican-related molecules.

A and B. Western blot analysis with 3F8 polyclonal anti-phosphacan (A) and with polyclonal antibodies against recombinant versican (B). Fractions 20, 22, 24, 26, 28, 30, 32, and 34 (lanes 1-8) from DEAE chromatophy were subjected to SDS-PAGE

(6-16% acrylamide gradient) under reducing conditions. C, D, and E. Western blot analysis with 473 anti-CS antibody (C), 3F8 polyclonal anti-phosphacan (D) and polyclonal anti-recombinant versican (E). Myelin extracts (lane 1), pooled DEAE chromatographic fractions 20-34 (lane 2), pooled flow-through from the PNA affinity column (lane 3), and pooled eluates from PNA affinity column (lane 4) were subjected to SDS-PAGE (6-16% acrylamide gradient) as in A and B. The position and molecular weight in kDa of marker proteins is indicated.

Figure 5. Analysis of the 70 kDa CSPGs after chondroitinase ABC treatment. Pooled eluates from the PNA affinity column (lane 1) and chondroitinase ABC treated pooled eluates from PNA affinity column (lane 2) were subjected to SDS-PAGE (6-16% acrylamide gradient) under reducing conditions and detected by amido black staining (A) and by Western blots with polyclonal anti-phosphacan 3F8 (B). A bands at 28 kDa (in A. lane 1) is PNA (artifactually eluted). Two bands above 72 kDa (in A. lane 2) are chondrontinase ABC. The position and molecular weight in kDa of marker proteins is indicated.

Figure 6. Determination of cell-type expression of the 70 kDa CSPGs. Total membrane proteins (100 ≒ g) from brain (lane 1), myelin (lane 2), oligodendrocytes (lane 3), astrocytes (lane 4), cerebellar neurons (lane 5), hippocampal neurons (lane 6), NG 108-15 cells (lane 7), and L-cells (lane 8) were subjected to SDS-PAGE (6-16% acrylamide gradient) under reducing conditions and detected by Western blots with polyclonal anti-phosphacan 3F8. The position and molecular weight in kDa of marker proteins is indicated.

Figure 7. Inhibitory effects of the 70 kDa CSPGs on neurite outgrowth from cerebellar neurons.
Cerebellar neurons were plated as single cell suspensions on the 70 kDa CSPGs (arretin) and other substrates applied to PORN-treated nitrocellulose substrates. Cells were maintained for 24 h before fixation and staining with toluidine blue. Error bars

indicate standard deviation. Coating concentrations were about 50 nM (1:25 dilution) and 10 nM (1:125 dilution) for arretin and denatured arretin (DN) and 10 nM for laminin. Bars represent percent neurons with neurites (mean ± SD).

Figure 8. Inhibitory effects of the 70 kDa CSPGs on neurite outgrowth from hippocampal neurons. Hippocampal neurons were plated as single cell suspensions on the 70 kDa CSPGs (arretin) and other substrates applied to PORN-treated tissue culture plastic. Cells were maintained for 24 h before fixation and staining with toluidine blue. Error bars indicate standard deviation. Coating concentrations were about 50 nM (1:25 dilution) and 10 nM (1:125 dilution) for arretin and denatured arretin (DN) and 10 nM for laminin. Bars represent percent neurons with neurites (mean ± SD).

Figure 9. inhibitory effects of the 70 kDa CSPGs on neurite outgrowth from NG108-15 cells.
NG108 cells were plated as single cell suspensions on the 70 kDa CSPGs (arretin; inhib.p) and other substrates applied to PLL-treated tissue culture plastic. Cells were maintained for 24 h before fixation and staining with toluidine blue. Coating concentrations were about 50 nM (1:5 dilution) for arretin (inhib.p) and denature arretin (denat. inhib.p) and 10 nM for laminin (LM). Bars represent neurons with neurites (% growth). PLL= polylysine.

Figure 10. SDS-PAGE showing purification of arretin. The polypeptide was visualized by dyes after gel electrophoresis. Lane 1 shows arretin purified by peanut agglutinin (PNA) affinity chromatography. Two bands at approximately 70kDa are visible. A band at 28 kDa was identified as a peanut agglutinin contaminant. Lane 2 shows pooled fractions from a DEAE chromatographic column that were applied to the PNA column for further purification of the arretin bands. Lane 3 shows myelin starting material from which arretin was extracted. Lane 4 shows molecular weight markers.

Figure 11. Two-dimensional gel electrophoresis separation of arretin obtained from

PNA column chromatography. Polypeptides were separated in the first dimension by isoelectric focusing followed by SDS-PAGE separation according to size in the second dimension. Spots 1,2, and 3 at approximately 70 kDa are separated from each other by size and charge. The spot at 28 kDa is peanut agglutinin, verified by Western blotting (not shown).

Figure 12. Anti-arretin antibody 18D2 neutralizes neurite outgrowth inhibition and cell body repulsion by arretin on NG 108-15 cells. Picture A demonstrates cells growing normally on a substrate of arretin-polylysine overlaid with anti-arretin 18D2. Picture B shows cell growth is inhibited on a substrate of arretin-polylysine treated with control antiserum.

Figure 13. Western blot showing that culture supernatant from monoclonal antibody 18D2 recognizes the approximately 70 kDa arretin component. Lane 1 (arros) shows partially purified arretin. Lane 2 shows myelin. Lane 3 shows octylglucoside/salt extract of myelin.

Figure 14. Growth cone collapse by arretin. A. Collapsed growth cones (arrows) after addition of arretin. B. Growth cones treated with DMEM as a control remain spread. Explants of P2 rat dorsal root ganglion neurons were plated on laminin can cultured overnight to allow neurite extension. Arretin purified by lectin chromatography (A) or control medium (B) was added to the cultures. The cultures were fixed with paraformaldehyde 30 min. later and viewed by phase contrast microcopy. The numbers of collapsed growth cones were counted. Arretin caused significantly more growth cone collapse than the PBS or DMEM controls.

DETAILED DESCRIPTION OF THE INVENTION

For the purpose of the present invention the following terms are defined below.

The term, neurite growth regulatory factor, refers to either arretin or its receptor.

"Agonist" refers to a pharmaceutical agent having biological activity of inhibiting the neurite outgrowth of neurons cultured on a permissive substrate or inhibiting the regeneration of damaged neurons. It would be desirable to inhibit neuron growth in cases of epilepsy, neuroblastoma, and neuromas, a disease state in a mammal which includes neurite outgrowth or other neural growth of an abnormal sort which causes pain at the end of an amputated limb. Antagonists which may be used in accordance with the present invention include without limitation a arretin fragment, an analog of arretin of the arretin fragment, a derivative of either arretin, the arretin fragment or said analog, an anti-idiotypic arretin antibody or a binding fragment thereof, arretin ectodomain and a pharmaceutical agent.

"Antagonist" refers to a pharmaceutical agent which in accordance with the present invention which inhibits at least on biological activity normally associate with arretin, that is blocking or suppressing the inhibition of neuron growth. Antagonists which may be used in accordance with the present invention include without limitation a arretin antibody or a binding fragment of said antibody, a arretin fragment, a derivative of arretin or of a arretin fragment, an analog of arretin or of a arretin fragment or of said derivative, and a pharmaceutical agent, and is further characterized by the property of suppressing arretin-mediated inhibition of neurite outgrowth.

An arretin antagonist is therefore, a chemical compound possessing the ability to alter the biological activity of the neuronal receptor for arretin such that growth of neurons or their axons is suppressed. The agonist or antagonist of arretin in accordance with the present invention is not limited to arretin or its derivatives, but also includes the therapeutic application of all agents, referred herein as pharmaceutical agents, which alter the biological activity of the neuronal receptor for arretin such that growth of neurons or their axon is suppressed. The receptor can be identified with know technologies by those skilled in the art (Mason, (1994) *Curr. Biol.*, 4:1158-1161) and

its association with arretin or fragments thereof can be determined. The neuronal receptor for arretin may or may not be the same as cell surface molecules that recognize and bind arretin in an adhesion assay (Kelm et al., (1994) *Curr. Biol.*, 4:965-972). Once the active arretin-recognition domain of the receptor(s) is/are known, appropriate peptides or their analogs can be designed and prepared to serve as agonist or antagonist of the arretin-receptor interaction.

The term "effective amount" or "growth-inhibiting amount" refers to the amount of pharmaceutical agent required to produce a desired agonist or antagonist effect of the arretin biological activity. The precise effective amount will vary with the nature of pharmaceutical agent used and may be determined by one or ordinary skill in the art with only routine experimentation.

As used herein, the terms "arretin biological activity" refers to cellular events triggered by arretin, being of either biochemical or biophysical nature. The following list is provided, without limitation, which discloses some of the known activities associated with contact-mediated growth inhibition of neurite outgrowth, adhesion to neuronal cells, and promotion of neurite out growth from new born dorsal root ganglion neurons.

Use of the phrase "substantially pure" or "isolated" in the present specification and claims as a modifier of DNA, RNA, polypeptides or proteins means that the DNA, RNA, polypeptides or proteins so designated have been separated from their in vivo cellular environment. As a result of this separation and purification, the substantially pure DNAs, RNAs, polypeptides and proteins are useful in ways that the non-separated, impure DNAs, RNAs, polypeptides or proteins are not.

As used herein, the term "biologically active", or reference to the biological activity of arretin or, or polypeptide fragment thereof, refers to a polypeptide that is able to produce one of the functional characteristics exhibited by arretin or its receptors

described herein. In one embodiment, biologically active proteins are those that demonstrate inhibitory growth activities central nervous system neurons. Such activity may be assayed by any method known to those of skill in the art.

Based on the present evidence that arretin is a growth inhibitory protein in myelin, the means exist to identify agents and therapies that suppress arretin-mediated inhibition of nerve growth. Further, one can exploit the growth inhibiting properties of arretin, or arretin agonists, to suppress undesired nerve growth. Without the critical finding that arretin has growth inhibitory properties, these strategies would not be developed.

The description of the present invention comprising a neuron and neural tumor growth regulatory system can be divided into the following sections solely for the purpose of description: (1) isolation, purification and characterization of arretin; (2) production of arretin-related derivatives, analogs, and peptides; (3) arretin antagonists and assay methods to identify arretin antagonists; (4) characterization of arretin receptors; (5) molecular cloning of genes or gene fragments encoding arretin and its receptors; (6) generation of arretin related derivatives, analogs, and peptides; (7) production of antibodies against the components of the arretin growth regulatory system, (ie. arretin, its receptors, and the nucleic acid sequences coding for these proteins); (8) the diagnostic, therapeutic and research uses for each of these components and the antibodies directed thereto.

1. Isolation, Purification, and Characterization of Arretin

The present invention relates to CNS myelin associated inhibitory proteins of neurite growth and receptors of CNS myelin associated inhibitory proteins of neurite growth. The CNS myelin associated inhibitory proteins of the invention may be isolated by first isolating myelin and subsequent purification therefrom. Isolation procedures which may be employed are described more fully in the sections which follow. Alternatively, the CNS myelin associated inhibitory proteins may be obtained from a recombinant

expression system. Procedures for the isolation and purification of receptors for the CNS myelin associated inhibitory proteins are described below.

Isolation and Purification of Arretin Proteins

Arretin proteins can be isolated from the CNS myelin of higher vertebrates including, but not limited to, birds or mammals (both human and nonhuman such as bovine, rat, porcine, chick, etc.). Myelin can be obtained from the optic nerve or from central nervous system tissue that includes but is not limited to spinal cords or brain stems. The tissue may be homogenized using procedures described in the art (Colman et al., 1982, J. Cell Biol. 95:598-608). The myelin fraction can be isolated subsequently also using procedures described (Colman et al., 1982, supra).

In one embodiment of the invention, the CNS myelin associated inhibitory proteins can be solubilized in detergent (for e.g., see McKerracher et al., 1994). The solubilized proteins can subsequently be purified by various procedures known in the art, including but not limited to
chromatography (e.g., ion exchange, affinity, and sizing chromatography), centrifugation, electrophoretic procedures, differential solubility, or by any other standard technique for the purification of proteins. In one aspect, the solubilized proteins can be subjected to one or two-dimensional electrophoresis, followed by elution from the gel. Gel-eluted proteins can be further purified and/or used to generate antibodies.

Alternatively, the CNS myelin associated inhibitory proteins may be isolated and purified using immunological procedures. For example, in one embodiment of the invention, the proteins can first be solubilized using detergent. The proteins may then be isolated by immunoprecipitation with antibodies. Alternatively, the CNS myelin associated inhibitory proteins may be isolated using immunoaffinity chromatography in which the proteins are applied to an antibody column in

solubilized form.

2. Production of Arretin-Related Derivatives, Analogs, and Peptides

The production and use of derivatives, analogs, and peptides related to arretin are also envisioned, and within the scope of the present invention and include molecules antagonistic to neurite growth regulatory factors (for example, and not by way of limitation, anti-idiotype antibodies). Such derivatives, analogs, or peptides which have the desired inhibitory activity can be used, for example, in the treatment of neuroblastoma. Derivatives, analogs, or peptides related to a neurite growth regulatory factor can be tested for the desired activity by assays for nonpermissive substrate effects or for growth cone collapse.

The neurite growth regulatory factor-related derivatives, analogs, and peptides of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, a cloned neurite growth regulatory factor gene can be modified by any of numerous strategies known in the art (Maniatis, et al., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). A given neurite growth regulatory factor sequence can be cleaved at appropriate sites with restriction endonuclease(s), subjected to enzymatic modifications if desired, isolated, and ligated in vitro. In the production of a gene encoding a derivative, analogue, or peptide related to a neurite growth regulatory factor, care should be taken to ensure that the modified gene remains within the same translational reading frame as the neurite growth regulatory factor, uninterrupted by translational stop signals, in the gene region where the desired neurite growth regulatory factor-specific activity is encoded.

Additionally, a given neurite growth regulatory factor gene can be mutated in vitro or in vivo, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or

destroy preexisting ones, to facilitate further in vitro modification. Any technique for mutagenesis known in the art can be used, including but not limited to, in vitro site-directed mutagenesis (Hutchinson, et al., 1978, J. Biol. Chem. 253:6551), use of TAB.RTM. linkers (Pharmacia), etc.

3. Arretin Antagonists and Assay Methods to Identify Arretin Antagonists

In one embodiment suitable as arretin antagonist candidates are developed comprising fragments, analogs and derivatives of arretin. Such candidates may interfere with arretin-mediated growth inhibition as competitive but non-functional mimics of endogenous arretin. From the amino acid sequence of arretin and from the cloned DNA coding for it, it will be appreciated that arretin fragments can be produced either by peptide synthesis or by recombinant DNA expression of either a truncated domain of arretin, or of intact arretin could be prepared using standard recominant procedures, that can then be digested enzymically in either a random or a site-selective manner. Analogs of arretin or arretin fragments can be generated also by recombinant DNA techniques or by peptide synthesis, and will incorporate one or more, e.g. 1-5, L- or D-amino acid substitutions. Derivatives of arretin, arretin fragments and arretin analogs can be generated by chemical reaction of the parent substance to incorporate the desired derivatizing group, such as N-terminal, C-terminal and intra-residue modifying groups that have the effect of masking or stabilizing the substance or target amino acids within it.

In specific embodiments of the invention, candidate arretin antagonists include those that are derived from a determination of the functionally active region(s) of arretin. The antibodies mentioned above and any others to be prepared against epitopes in arretin, when found to be function-blocking in *in vitro* assays, can be used to map the active regions of the polypeptide as has been reported for other proteins (for example, see Fahrig et al., (1993) *Europ., J. Neurosci.,* 5: 1118-1126; Tropak et al., (1994) *J.*

Neurochem., 62: 854-862). Thus, it can be determined which regions of arretin are recognized by neuronal receptors and/or are involved in inhibition of neurite outgrowth. When those are known, synthetic peptides can be prepared to be assayed as candidate antagonists of the arretin effect. Derivatives of these can be prepared, including those with selected amino acid substitutions to provide desirable properties to enhance their effectiveness as antagonists of the arretin candidate functional regions of arretin can also be determined by the preparation of altered forms of the arretin domains using recombinant DNA technologies to produce deletion or insertion mutants that can be expressed in various cell types as chimaeric proteins that contain the Fc portion of immunoglobulin G (Kelm et al., (1994) *Curr. Biol.*, 4: 965-972). Alternatively, candidate mutant forms of arretin can be expressed on cell surfaces by transfection of various cultured cell types. All of the above forms of arretin, and forms that may be generated by technologies not limited to the above, can be tested for the presence of functional regions that inhibit or suppress neurite outgrowth, and can be used to design and prepare peptides to serve as antagonists.

In accordance with an aspect of the invention, the arretin antagonist is formulated as a pharmaceutical composition which contains the arretin antagonist in an amount effective to suppress arretin-mediated inhibition of nerve growth, in combination with a suitable pharmaceutical carrier. Such compositions are useful, in accordance with another aspect of the invention, to suppress arretin-inhibited nerve growth in patients diagnosed with a variety of neurological disorder, conditions and ailments of the PNS and the CNS where treatment to increase neurite extension, growth, or regeneration is desired, e.g., in patients with nervous system damage. Patients suffering from traumatic disorders (including but not limited to spinal cord injuries, spinal cord lesions, surgical nerve lesions or other CNS pathway lesions) damage secondary to infarction, infection, exposure to toxic agents, malignancy, paraneoplastic syndromes, or patients with various types of degenerative disorders of the central nervous system (Cutler, (1987) In: *Scientific American Medicines*, vol. 2, Scientific American Inc., N.Y., pp. 11-1-11-13) can be treated with such arretin antagonists. Examples of such

disorders include but are not limited to Strokes, Alzheimer's disease, Down's syndrome, Creutzfeldt-Jacob disease, kuru, Gerstman-Straussler syndrome, scrapie, transmissible mink encephalopathy, Huntington's disease, Riley-Day familial dysautonomia, multiple system atrophy, amylotrophic lateral sclerosis or Lou Gehrig's disease, progressive supranuclear palsy, Parkinson's disease and the like. The arretin antagonists may be used to promote the regeneration of CNS pathways, fiber systems and tracts. Administration of antibodies directed to an epitope of arretin, or the binding portion thereof, or cells secreting such antibodies can also be used to inhibit arretin function in patients. In a particular embodiment of the invention, the arretin antagonist is used to promote the regeneration of nerve fibers over long distances following spinal cord damage.

In another embodiment, the invention provides an assay method adapted to identify arretin antagonists, that is agents that block or suppress the growth-inhibiting action of arretin. In its most convenient form, the assay is a tissue culture assay that measures neurite out-growth as a convenient end-point, and accordingly uses nerve cells that extend neurites when grown on a permissive substrate. Nerve cells suitable in this regard include neuroblastoma cells of the NG108 lineage, such as NG108-15, as well as other neuronal cell lines such as PC12 cells (American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852 USA, ATCC accession NO. CRL 1721), human neuroblastoma cells, and primary cultures of CNS or PNS neurons taken from embryonic, postnatal or adult animals. The nerve cells, for instance about 10^3 cells-microwell or equivalent, are cultured on a growth permissive substrate, such as polylysine or laminin, that is over-layed with a growth-inhibiting amount of arretin. The arretin incorporated in the culture is suitably myelin-extracted arretin, although forms of arretin other than endogenous forms can be used provided they exhibit the arretin property of inhibiting neuron growth when added to a substrate that is otherwise growth permissive.

In this assay, candidate arretin antagonists, *i.e.*, compounds that block the growth-

inhibiting effect of arretin, are added to the arretin-containing tissue culture preferably in amount sufficient to neutralize the arretin growth-inhibiting activity, that is between 1.5 and 15 μg of arretin antagonists per well containing a density of 1000 NG108-15 cells/well cultured for 24 hr. in Dulbecco's minimal essential medium. After culturing for a period sufficient for neurite outgrowth, e.g. 3-7 days, the culture is evaluated for neurite outgrowth, and arretin antagonists are thereby revealed as those candidates which elicit neurite outgrowth. Desirably, candidates selected as arretin antagonists are those which elicit neurite outgrowth to a statistically significant extent compared to neurons plated on arretin alone.

Screening for compounds that stimulate cell adhesion and neurite growth on arretin-coated substrates.

Arretin not only prevents neurite growth but also reduces the adhesion of cells to the substrate. Since cell adhesion is technically far easier to assay quantitatively than neurite growth, cell adhesion can be used as a first screen for high-through-put screening of a large number of compounds. This can be done using the MTT [3{4-5-dimethylthiazol-2-yl]-2,5-diphenyltertrazolium bromide) assay. MTT is taken up by live cells and converted by the mitochondria into a blue substrate that can be quantified by a densitometer. For this assey, 96-well plates are coated with arretin. After washing wells the add chemical compounds can be added to the well for 1-2 hours or along with neuronal cells such as NG108-15 cells. After 2-4 hours or overnight incubation with the cells, the cultures are washed to remove unattached cells.
MTT is then added to the cells at a concentration of 0.5mg/ml in culture medium. Incubate for 4 hours at 37oC in a 5% CO_2 incubator. Wash once with PBS and add acid isopropanol (100ul/well), and mix with a pipette. After 5 minutes the plates are read with ELISA reader at 550nm.

Other assay tests that could be used include without limitation the following: 1) The growth cone collapse assay that is used to assess growth inhibitory activity of collapsin

(Raper, J.A., and Kapfhammer, J.P., (1990) *Neuron*, 2:21-29; Luo et al., (1993) *Cell*, 75:217-227) and of various other inhibitory molecules (Igarashi, M. et al., (1993) *Science*, 259:77-79) whereby the test substance is added to the culture medium and a loss of elaborate growth cone morphology is scored. 2) The use of patterned substrates to assess substrate preference (Walter, J. et al., (1987) *Development*, 101:909-913; Stahl et al., (1990) *Neuron*, 5:735-743) or avoidance of test substrates (Ethell, D.W. et al., (1993) *Dev. Brain Res.*, 72:1-8). 3) The expression of recombinant proteins on a heterologous cell surface, and the transfected cells are used in co-culture experiments. The ability of the neurons to extend neurites on the transfected cells is assessed (Mukhopadhyay et al., (1994) *Neuron*, 13:757-767). 4) The use of sections of tissue, such as sections of CNS white matter, to assess molecules that may modulate growth inhibition (Carbonetto et al., (1987) *J. Neuroscience*, 7:610-620; Savlo, T. and Schwab, M.E., (1989) *J. Neurosci.*, 9:1126-1133). 5) Neurite retraction assays whereby test substrates are applied to differentiated neural cells for their ability to induce or inhibit the retraction of previously extended neurites (Jalnink et al., (1994) *J. Cell Bio.*, 126:801-810; Sudan, H.S. et al., (1992) *Neuron*, 8:363-375; Smalheiser, N. (1993) *J. Neurochem.*, 61:340-342). 6) The repulsion of cell-cell interactions by cell aggregation assays (Kelm, S. et al., (1994) *Current Biology*, 4:965-972; Brady-Kainay, S. et al., (1993) *J. Cell Biol.*, 4:961-972). 7) The use of nitrocellulose to prepare substrates for growth assays to assess the ability of neural cells to extend neurites on the test substrate (Laganeur, C. and Lemmon, V., (1987)*PNAS*, 84:7753-7757; Dou, C-L and Levine, J.M., (1994) *J. Neuroscience*, 14:7616-7628).

Useful arretin antagonists include antibodies to arretin and the binding fragments of those antibodies. Antibodies which are either monoclonal or polyclonal can be produced which recognize arretin and its various epitopes using now routine procedures. For the raising of antibody, various host animals can be immunized by injection with arretin or fragment thereof, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and

incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinmitrophenol, and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin).

4. Isolation and Purification of Receptors for Arretin

Receptors for arretin can be isolated from cells whose attachment, spreading, growth and/or motility is inhibited by arretin. Such cells include but are not limited to fibroblasts and neurons. In a preferred embodiment, neurons are used as the source for isolation and purification of the receptors.

In one embodiment, receptors to arretin may be isolated by affinity chromatography of neuronal plasma membrane fractions, in which a myelin associated inhibitory protein or peptide fragment thereof is immobilized to a solid support. Alternatively, receptor cDNA may be isolated by expression cloning using purified arretin as a ligand for the selection of receptor-expressing clones.

Alternatively, arretin protein may be tagged for use as a reporter to detect receptors of arretin, using techniques that are well known in the art. There are many different types of tags that may be employed such as flourescence radioactive tags.

5. Molecular Cloning of Genes or Gene Fragments Encoding Arretin and Its Receptors

Any mammalian cell can potentially serve as the nucleic acid source for the molecular cloning of the genes encoding arretin or its receptors. The DNA may be obtained by standard procedures known in the art from cloned DNA (e.g., a DNA "library"), by chemical synthesis, by cDNA cloning, or by the cloning of genomic DNA, or fragments

thereof, purified from the desired mammalian cell. (See, for example, Maniatis et al., 1982, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.; Glover, D. M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U. K., Vol. I, II.) Clones derived from genomic DNA may contain regulatory and intron DNA regions, in addition to coding regions; clones derived from cDNA will contain only exon sequences. Whatever the source, a given neurite growth regulatory factor gene should be molecularly cloned into a suitable vector for propagation of the gene.

In the molecular cloning of a neurite growth regulatory factor gene from genomic DNA, DNA fragments are generated, some of which will encode the desired neurite growth regulatory factor gene. The DNA may be cleaved at specific sites using various restriction enzymes. Alternatively, one may use DNAse in the presence of manganese to fragment the DNA, or the DNA can be physically sheared, as for example, by sonication. The linear DNA fragments can then be separated according to size by standard techniques, including but not limited to, agarose and polyacrylamide gel electrophoresis and column chromatography.

Once the DNA fragments are generated, identification of the specific DNA fragment containing a neurite growth regulatory factor gene may be accomplished in a number of ways. For example, if an amount of a neurite growth regulatory factor gene or its specific RNA, or a fragment thereof, is available and can be purified and labeled, the generated DNA fragments may be screened by nucleic acid hybridization to the labeled probe (Benton and Davis, 1977, Science 196:180; Grunstein and Hogness, 1975, Proc. Natl. Acad. Sci. U.S.A. 72:3961-3965). For example, in a preferred embodiment, a portion of a neurite growth regulatory factor amino acid sequence can be used to deduce the DNA sequence, which DNA sequence can then be synthesized as an oligonucleotide for use as a hybridization probe. Alternatively, if a purified neurite growth regulatory factor probe is unavailable, nucleic acid fractions enriched in neurite growth regulatory factor may be used as a probe, as an initial selection procedure. It is

also possible to identify an appropriate neurite growth regulatory factor-encoding fragment by restriction enzyme digestion(s) and comparison of fragment sizes with those expected according to a known restriction map if such is available. Further selection on the basis of the properties of the gene, or the physical, chemical, or immunological properties of its expressed product, as described above, can be employed after the initial selection.

A neurite growth regulatory factor gene can also be identified by mRNA selection using nucleic acid hybridization followed by in vitro translation or translation in Xenopus oocytes. In an example of the latter procedure, oocytes are injected with total or size fractionated CNS mRNA populations, and the membrane-associated translation products are screened in a functional assay (3T3 cell spreading). Preadsorption of the RNA with complementary DNA (cDNA) pools leading to the absence of expressed inhibitory factors indicates the presence of the desired cDNA. Reduction of pool size will finally lead to isolation of a single cDNA clone. In an alternative procedure, DNA fragments can be used to isolate complementary mRNAs by hybridization. Such DNA fragments may represent available, purified neurite growth regulatory factor DNA, or DNA that has been enriched for neurite growth regulatory factor sequences. Immunoprecipitation analysis or functional assays of the in vitro translation products of the isolated mRNAs identifies the mRNA and, therefore, the cDNA fragments that contain neurite growth regulatory factor sequences. An example of such a functional assay involves an assay for nonpermissiveness in which the effect of the various translation products on the spreading of 3T3 cells on a polylysine coated tissue culture dish is observed. In addition, specific mRNAs may be selected by adsorption of polysomes isolated from cells to immobilized antibodies specifically directed against a neurite growth regulatory factor protein. A radiolabeled neurite growth regulatory factor cDNA can be synthesized using the selected mRNA (from the adsorbed polysomes) as a template. The radiolabeled mRNA or cDNA may then be used as a probe to identify the neurite growth regulatory factor DNA fragments from among other genomic DNA fragments.

Alternatives to isolating the neurite growth regulatory factor genomic DNA include, but are not limited to, chemically synthesizing the gene sequence itself from a known sequence or making cDNA to the mRNA which encodes the neurite growth regulatory factor gene. Other methods are possible and within the scope of the invention. The identified and isolated gene or cDNA can then be inserted into an appropriate cloning vector. A large number of vector-host systems known in the art may be used. Possible vectors include, but are not limited to, cosmids, plasmids or modified viruses, but the vector system must be compatible with the host cell used. Such vectors include, but are not limited to, bacteriophages such as lambda derivatives, or plasmids such as pBR322 or pUC plasmid derivatives. Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, etc.

In an alternative embodiment, the neurite growth regulatory factor gene may be identified and isolated after insertion into a suitable cloning vector, in a "shot gun" approach. Enrichment for a given neurite growth regulatory factor gene, for example, by size fractionation or subtraction of cDNA specific to low neurite growth regulatory factor producers, can be done before insertion into the cloning vector. In another embodiment, DNA may be inserted into an expression vector system, and the recombinant expression vector containing a neurite growth regulatory factor gene may then be detected by functional assays for the neurite growth regulatory factor protein.

The neurite growth regulatory factor gene is inserted into a cloning vector which can be used to transform, transfect, or infect appropriate host cells so that many copies of the gene sequences are generated. This can be accomplished by ligating the DNA fragment into a cloning vector which has complementary cohesive termini. However, if the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules may be enzymatically modified. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition

sequences. In an alternative method, the cleaved vector and neurite growth regulatory factor gene may be modified by homopolymeric tailing. Identification of the cloned neurite growth regulatory factor gene can be accomplished in a number of ways based on the properties of the DNA itself, or alternatively, on the physical, immunological, or functional properties of its encoded protein. For example, the DNA itself may be detected by plaque or colony nucleic acid hybridization to labeled probes (Benton, W. and Davis, R., 1977, Science 196:180; Grunstein, M. and Hogness, D., 1975, Proc. Natl. Acad. Sci. U.S.A. 72:3961). Alternatively, the presence of a neurite growth regulatory factor gene may be detected by assays based on properties of its expressed product. For example, cDNA clones, or DNA clones which hybrid-select the proper mRNAs, can be selected which produce a protein that inhibits in vitro neurite outgrowth. If an antibody to a neurite growth regulatory factor is available, a neurite growth regulatory factor protein may be identified by binding of labeled antibody to the putatively neurite growth regulatory factor-synthesizing clones, in an ELISA (enzyme-linked immunosorbent assay)-type procedure. In specific embodiments, transformation of host cells with recombinant DNA molecules that incorporate an isolated neurite growth regulatory factor gene, cDNA, or synthesized DNA sequence enables generation of multiple copies of the gene. Thus, the gene may be obtained in large quantities by growing transformants, isolating the recombinant DNA molecules from the transformants and, when necessary, retrieving the inserted gene from the isolated recombinant DNA. If the ultimate goal is to insert the gene into virus expression vectors such as vaccinia virus or adenovirus, the recombinant DNA molecule that incorporates a neurite growth regulatory factor gene can be modified so that the gene is flanked by virus sequences that allow for genetic recombination in cells infected with the virus so that the gene can be inserted into the viral genome. After the neurite growth regulatory factor DNA-containing clone has been identified, grown, and harvested, its DNA insert may be characterized as described herein. When the genetic structure of a neurite growth regulatory factor gene is known, it is possible to manipulate the structure for optimal use in the present invention. For example, promoter DNA may be ligated 5' of a neurite growth regulatory factor coding sequence,

in addition to or replacement of the native promoter to provide for increased expression of the protein. Many manipulations are possible, and within the scope of the present invention.

Expression of the Cloned Neurite Growth Regulatory Factor Genes.

The nucleotide sequence coding for a neurite growth regulatory factor protein or a portion thereof, can be inserted into an appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. The necessary transcriptional and translation signals can also be supplied by the native neurite growth regulatory factor gene and/or its flanking regions. A variety of host-vector systems may be utilized to express the protein-coding sequence. These include but are not limited to mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA. The expression elements of these vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used.Any of the methods previously described for the insertion of DNA fragments into a vector may be used to construct expression vectors containing a chimeric gene consisting of appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include in vitro recombinant DNA and synthetic techniques and in vivo recombinations (genetic recombination).

Expression vectors containing neurite growth regulatory factor gene inserts can be identified by three general approaches: (a) DNA-DNA hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a foreign gene inserted in an expression vector can be detected by DNA-DNA hybridization using probes comprising sequences that are

homologous to an inserted neurite growth regulatory factor gene. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign genes in the vector. For example, if a given neurite growth regulatory factor gene is inserted within the marker gene sequence of the vector, recombinants containing the neurite growth regulatory factor insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the foreign gene product expressed by the recombinant. Such assays can be based on the physical, immunological, or functional properties of a given neurite growth regulatory factor gene product.

Once a particular recombinant DNA molecule is identified and isolated, several methods known in the art may be used to propagate it. Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. As
previously explained, the expression vectors which can be used include, but are not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (e.g., lambda), and plasmid and cosmid DNA vectors, to name but a few.

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus, expression of the genetically engineered neurite growth regulatory factor protein may be controlled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (e.g., glycosylation, cleavage) of proteins. Appropriate cell lines or host

systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system can be used to produce an unglycosylated core protein product. Expression in yeast will produce a glycosylated product. Expression in mammalian (e.g. COS) cells can be used to ensure "native" glycosylation of the heterologous neurite growth regulatory factor protein. Furthermore, different vector/host expression systems may effect processing reactions such as proteolytic cleavages to different extents.

Identification and Purification of the Expressed Gene Product

Once a recombinant which expresses a given neurite growth regulatory factor gene is identified, the gene product can be purified and analyzed as described above. The amino acid sequence of arretin and its receptor protein can be deduced from the nucleotide sequence of the cloned gene, allowing the protein, or a fragment thereof, to be synthesized by standard chemical methods known in the art (e.g., see Hunkapiller, et al., 1984, Nature 310:105-111). In particular embodiments of the present invention, such neurite growth regulatory factor proteins, whether produced by recombinant DNA techniques or by chemical synthetic methods, include but are not limited to those containing altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amno acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine, and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Also included within the scope of the invention are neurite growth regulatory factor proteins

which are differentially modified during or after translation, e.g., by glycosylation, proteolytic cleavage, etc.

Characterization of the Neurite, Growth Regulatory Factor Genes

The structure of a given neurite growth regulatory factor gene can be analyzed by various methods known in the art.

The cloned DNA or cDNA corresponding to a given neurite growth regulatory factor gene can be analyzed by methods including but not limited to Southern hybridization (Southern, 1975, J. Mol. Biol. 98:503-517), Northern hybridization (Alwine, et al., 1977, Proc. Natl. Acad. Sci. U.S.A. 74:5350-5354; Wahl, et al., 1987, Meth. Enzymol. 152:572-581), restriction endonuclease mapping (Maniatis, et al., 1982, Molecular loning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), and DNA sequence analysis. DNA sequence analysis can be performed by any techniques known in the art including but not limited to the method of Maxam and Gilbert (1980, Meth. Enzymol. 65:499-560), the Sanger dideoxy method (Sanger, et al., 1977, Proc. Natl. Acad. Sci. U.S.A. 74:5463-5467), or use of an automated DNA sequenator (e.g., Applied Biosystems, Foster City, Calif.).

6. *Production of Antibodies Against the Components of the Arretin Growth Regulatory System*

Antibodies can be produced which recognize neurite growth regulatory factors or related proteins. Such antibodies can be polyclonal or monoclonal.Various procedures known in the art may be used for the production of polyclonal antibodies to epitopes of a given neurite growth regulatory factor. For the production of antibody, various host animals can be immunized by injection with a neurite growth regulatory factor protein, or a synthetic protein, or fragment thereof, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response,

depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum. A monoclonal antibody to an epitope of a neurite growth regulatory factor can be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein (1975, Nature 256:495-497), and the more recent human B cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72) and EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In a particular embodiment, the procedure described . may be used to obtain mouse monoclonal antibodies which recognize arretin and its receptors.

The monoclonal antibodies for therapeutic use may be human monoclonal antibodies or chimeric human-mouse (or other species) monoclonal antibodies. Human monoclonal antibodies may be made by any of numerous techniques known in the art (.RTM..q., Teng et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:7308-7312; Kozbor et al., 1983, Immunology Today 4:72-79; Olsson et al., 1982, Meth. Enzymol. 92:3-16). Chimeric antibody molecules may be prepared containing a mouse antigen-binding domain with human constant regions (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851, Takeda et al., 1985, Nature 314:452). A molecular clone of an antibody to a neurite growth regulatory factor epitope can be prepared by known techniques. Recombinant DNA methodology (see e.g., Maniatis et al., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) may be used to construct nucleic acid sequences which encode a monoclonal antibody olecule, or antigen binding region thereof.

A monoclonal antibody to an epitope of arretin can be prepared by using any technique

which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Köler and Milstein ((1975) *Nature*, 256:495-497), and the more recent human B cell hybridoma technique (Kozbor et al., (1983) *Immunology Today,* 4:72) and EBV-hybridoma technique (Cole et al., (1985) In *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp 77-96). In a particular embodiment, the procedure described by Nobile-Orazio et al. ((1984) *Neurology,* 34:1336-1342) may be used to obtain antibodies which recognize recombinant Arretin (for example of techniques, see Attia S. et al., (1993) *J. Neurochem.,* 61: 718-726).

The monoclonal antibodies for therapeutic use may be human monoclonal antibodies or chimeric human-mouse (or other species) monoclonal antibodies. Human monoclonal antibodies may be made by any of numerous techniques known in the art (e.g. Tan et al., (1983) *Proc. Natl. Acad. Sci. U.S.A.,* 80: 7308-7312; Kozbor et al., (1983) *Immunology Today, 4: 72-79; Olsson et al.,* (1982) *Meth. Enzymol.,* 92: 3-16*).* Chimeric antibody molecules may be prepared containing a mouse antigen-binding domain with human contact regions (Morrision et al., (1984) *Proc. Natl. Acad. Sci. U.S.A.,* 81: 6851; Takeda et al., (1985) *Nature,* 314: 452).

A molecular clone of an antibody to a Arretin epitope can be prepared by known techniques. Recombinant DNA methodology may be used to construct nucleic acid sequences which encode a monoclonal antibody molecule, or antigen binding region thereof (see e.g., Maniatis et al., (1982) In *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.).

For use, arretin antibody molecules may be purified by known techniques, such as immunoabsorption or immunoaffinity chromatography, chromotographic methods such as HPLC (high performance liquid chromatography), or a combination thereof, etc.

Arretin antibody fragments which contain the idiotype of the molecule can be generated

by known techniques. For example, such fragments include but are not limited to: the F (ab')$_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab, fragments which cen be generated by reducing the disulfide bridges of the F (ab')$_2$ fragment, and the two Fab or Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

Monoclonal antibodies known to react with human arretin may be tested for their usefulness to serve as arretin antagonists (Nobile-Orazio et al., (1984) *Neurology,* 34: 1336-1342; Doberson et al., (1985) *Neurochem. Res.,* 10: 499-513).

Antibody molecules may be purified by known techniques, e.g., immunoabsorption or immunoaffinity chromatography, chromatographic methods such as HPLC (high performance liquid chromatography), or a combination thereof, etc.Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the F(ab').sub.2 fragment which can be produced by pepsin digestion of the antibody molecule; the Fab, fragments which can be generated by reducing the disulfide bridges of the F(ab').sub.2 fragment, and the 2 Fab or Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

7. Diagnostic, Therapeutic and Research Uses for each of these Components and the Antibodies Directed Thereto

Arretin, its receptors, analogs, derivatives, and subsequences thereof, and anti-inhibitory protein antibodies or peptides have uses in diagnostics. Such molecules can be used in assays such as immunoassays to detect, prognose, diagnose, or monitor various conditions, diseases, and disorders affecting neurite growth extension, invasiveness, and regeneration. In one embodiment of the invention, these molecules may be used for the diagnosis of malignancies. Alternatively, the CNS myelin associated inhibitory proteins, analogs, derivatives, and subsequences thereof and

antibodies thereto may be used to monitor therapies for diseases and conditions which ultimately result in nerve damage; such diseases and conditions include but are not limited to CNS trauma, (e.g. spinal cord injuries), infarction, infection, malignancy, exposure to toxic agents, nutritional deficiency, paraneoplastic syndromes, and degenerative nerve diseases (including but not limited to Alzheimer's disease, Parkinson's disease, Huntington's Chorea, amyotrophic lateral sclerosis, progressive supra-nuclear palsy, and other dementias). In a specific embodiment, such molecules may be used to detect an increase in neurite outgrowth as an indicator of CNS fiber regeneration. For example, in specific embodiments, the absence of the CNS myelin associated inhibitory proteins in a patient sample containing CNS myelin can be a diagnostic marker for the presence of a malignancy, including but not limited to glioblastoma, neuroblastoma, and melanoma, or a condition involving nerve growth, invasiveness, or regeneration in a patient. In a particular embodiment, the absence of the inhibitory proteins can be detected by means of an immunoassay in which the lack of any binding to anti-inhibitory protein antibodies is observed. The immunoassays which can be used include but are not limited to competitive and non-competitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, precipitation reactions, gel diffusion precipitation reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, immunoelectrophoresis assays, and immunohistochemistry on tissue sections, to name but a few.

In accordance with another aspect of the invention, arretin and related compounds that retain the arretin property of inhibiting neurone growth (herein referred to as arretin agonists) are used therapeutically to treat conditions in which suppression of undesirable neuronal growth is desired. These include for example the treatment of tumors of nerve tissue and of conditions resulting from uncontrolled nerve sprouting such as is associated with epilepsy and in the spinal cord after nerve injury. In one embodiment patients with neuroblastoma, and particularly with neuropathies associated

with circulating arretin antibody, can be treated with arretin or arretin agonist.

Useful for nerve growth suppression are pharmaceutical compositions that contain, in an amount effective to suppress nerve growth, either arretin or a arretin agonist in combination with an acceptable carrier. Arretin can be obtained either by extraction from myelin as described above or, more practically, by recombinant DNA expression of Arretin-encoding DNA, for example, in the manner reported for MAG by Attia S., *et al.*, J. Neurochem.,61, 718-726, 1993. Useful arretin agonists are those compounds which, when added to the permissive substrate described above, suppress the growth of neuronal cells. Particularly useful Arretin agonists are those compounds which cause a statistically significant reduction in the number of neuronal cells that extend neurites, relative to control cells not exposed to the agonist. Candidate Arretin agonists include fragments of Arretin that incorporate the ectodomain, including the ectodomain *per se* and other N- and/or C-terminally truncated fragments of Arretin or the ectodomain, as well as analogs thereof in which amino acids, e.g. from 1 to 10 residues, are substituted, particularly conservatively, and derivatives of Arretin or Arretin fragments in which the N- and/or C-terminal residues are derivatized by chemical stabilizing groups. Such Arretin agonists can also include anti-idiotypes of Arretin antibodies and their binding fragments.

In specific embodiments of the invention, candidate Arretin agonists include specific regions of the Arretin molecule, and analogs or derivatives of these. These can be identified by using the same technologies described above for identification of Arretin regions that serve as inhibitors of neurite outgrowth.

The Arretin related derivatives, analogs, and fragments of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, Arretin-encoding DNA can be modified by any of numerous strategies known in the art (Maniatis *et al.*, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold

Spring Harbor, N.Y., 1982), such as by cleavage at appropriate sites with restriction endonuclease(s), subjected to enzymatic modifications if desired, isolated, and ligated *in-vitro*.

Additionally, the Arretin-encoding gene can be mutated *in-vitro* or *in-vivo* for instance in the manner applied fro production of the ectodomain, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in-vitro* modification. Any technique for mutagenesis known in the art cab be used, including but not limited to, *in-vitro* site directed mutagenesis (Hutchinson, *et al.*, J. Biol. Chem., 253, 6551, 1978), use of TAB™ linkers (Pharmacia), etc.

For delivery of Arretin, Arretin agonist or Arretin antagonist, various known delivery systems can be used, such as encapsulation in liposmes or semipermeable membranes, expression in suitably transformed or transfection glial cells, oligodendroglial cells, fibroblasts, etc. according to the procedure known to those skilled in the are (Lindvall *et al.*, Curr. Opinion Neurobiol., 4, 752-757, 1994). Linkage to ligands such as antibodies can be used to target delivery to myelin and to other therapeutically relevant sites *in-vivo*. Methods of introduction include, but are not limited to, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, oral, and intranasal routes, and transfusion into ventricles or a site of operation (e.g. for spinal cord lesions) or tumor removal. Likewise, cells secreting Arretin antagonist activity, for example, and not by way of limitation, hybridoma cells encapsulated in a suitable biological membrane may be implanted in a patient so as to provide a continuous source of Arretin inhibitor.

In another specific embodiment, ligands which bind to arretin or its receptors can be used in imaging techniques. For example, small peptides (e.g., inhibitory protein receptor fragments) which bind to the inhibitory proteins, and which are able to

penetrate through the blood-brain barrier, when labeled appropriately, can be used for imaging techniques such as PET (positron emission tomography) diagnosis or scintigraphy detection, under conditions noninvasive to the patient.

Neurite growth inhibitory factor genes, DNA, cDNA, and RNA, and related nucleic acid sequences and subsequences, including complementary sequences, can also be used in hybridization assays. The neurite growth inhibitory factor nucleic acid sequences, or subsequences thereof comprising about at least 15 nucleotides, can be used as hybridization probes. Hybridization assays can be used to detect, prognose, diagnose, or monitor conditions, disorders, or disease states associated with changes in neurite growth inhibitory factor expression as described supra. For example, total RNA in myelin, e.g., on biopsy tissue sections, from a patient can beassayed for the presence of neurite growth inhibitory factor mRNA, where the amount of neurite growth inhibitory factor mRNA is indicative of the level of inhibition of neurite outgrowth activity in a given patient.

Therapeutic Uses of Arretin

CNS myelin associated inhibitory proteins of the present invention can be therapeutically useful in the treatment of patients with malignant tumors including, but not limited to melanoma or tumors of nerve tissue (e.g. neuroblastoma). In one embodiment, patients with neuroblastoma can be treated with arretin or analogs, derivatives, or subsequences thereof, and the human functional equivalents thereof, which are inhibitors of neurite extension.

In an alternative embodiment, derivatives, analogs, or subsequences of CNS myelin inhibitory proteins which inhibit the native inhibitory protein function can be used in regimens where an increase in neurite extension, growth, or regeneration is desired, e.g., in patients with nervous system damage. Patients suffering from traumatic disorders (including but not limited to spinal cord injuries, spinal cord lesions, or other

CNS pathway lesions), surgical nerve lesions, damage secondary to infarction, infection, exposure to toxic agents, malignancy, paraneoplastic syndromes, or patients with various types of degenerative disorders of the central nervous system (Cutler, 1987, In: Scientific American Medicines v. 2, Scientific American Inc., N.Y., pp. 11-1-11-13) can be treated with such inhibitory protein antagonists. Examples of such disorders include but are not limited to Alzheimer's Disease, Parkinsons' Disease, Huntington's Chorea, amyotrophic lateral sclerosis, progressive supranuclear palsy and other dementias. Such antagonists may be used to promote the regeneration of CNS pathways, fiber systems and tracts. Administration of antibodies directed to an epitope of, (or the binding portion thereof, or cells secreting such as antibodies) can also be used to inhibit arretin protein function in patients. In a particular embodiment of the invention, antibodies directed to arretin may be used to promote the regeneration of nerve fibers over long distances following spinal cord damage.

Various delivery systems are known and can be used for delivery of arretin, related molecules, or antibodies thereto, e.g., encapsulation in liposomes or semipermeable membranes, expression by bacteria, etc. Linkage to ligands such as antibodies can be used to target myelin associated protein-related molecules to therapeutically desirable sites in vivo. Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, oral, and intranasal routes, and infusion into ventricles or a site of operation (e.g. for spinal cord lesions) or tumor removal. Likewise, cells secreting CNS myelin inhibitory protein antagonist activity, for example, and not by way of limitation, hybridoma cells, encapsulated in a suitable biological membrane may be implanted in a patient so as to provide a continuous source of anti-CNS myelin inhibiting protein antibodies.

In addition, any method which results in decreased synthesis of arretin or its receptors may be used to diminish their biological function. For example, and not by way of limitation, agents toxic to the cells which synthesize arretin and/or its receptors (e.g. oligodendrocytes) may be used to decrease the concentration of inhibitory proteins to

promote regeneration of neurons.

Arretin Receptors

Arretin receptors as well as analogs, derivatives, and subsequences thereof, and anti-receptor antibodies have uses in diagnostics. These molecules of the invention can be used in assays such as immunoassays or binding assays to detect, prognose, diagnose, or monitor various conditions, diseases, and disorders affecting neurite growth, extension, invasion, and regeneration. For example, it is possible that a lower level of expression of these receptors may be detected in various disorders associated with enhanced neurite sprouting and plasticity or regeneration such as those involving nerve damage, infarction, degenerative nerve diseases, or malignancies. The CNS myelin associated inhibitory protein receptors, analogs, derivatives, and subsequences thereof may also be used to monitor therapies for diseases and disorders which ultimately result in nerve damage, which include but are not limited to CNS trauma (e.g. spinal cord injuries), stroke, degenerative nerve diseases, and for malignancies.

The assays which can be used include but are not limited to those described above.

Arretin receptor genes and related nucleic acid sequences and subsequences, including complementary sequences, can also be used in hybridization assays, to detect, prognose, diagnose, or monitor conditions, disorders, or disease states associated with changes in neurite growth inhibitory factor receptor expression.

Arretin Receptors

Arretin receptors or fragments thereof, and antibodies thereto, can be therapeutically useful in the treatment of patients with nervous system damage including but not limited to that resulting from CNS trauma (e.g., spinal cord injuries), infarction, or degenerative disorders of the central nervous system which include but are not limited

to Alzheimer's disease, Parkinson's disease, Huntington's Chorea, amyotrophic lateral sclerosis, or progressive supranuclear palsy. For example, in one embodiment, arretin receptors, or subsequences or analogs thereof which contain the inhibitory protein binding site, can be administered to a patient to "compete out" binding of the inhibitory proteins to their natural receptor, and to thus promote nerve growth or regeneration in the patient. In an alternative embodiment, antibodies to the inhibitory protein receptor (or the binding portion thereof or cells secreting antibodies binding to the receptor) can be administered to a patient in order to prevent receptor function and thus promote nerve growth or regeneration in the patient. Patients in whom such a therapy may be desired include but are not limited to those with nerve damage, stroke, or degenerative disorders of the central nervous system as described supra.

Various delivery systems are known and can be used for delivery of arretin receptors, related molecules, or antibodies thereto, e.g., encapsulation in liposomes, expression by bacteria, etc. Linkage to ligands such as antibodies can be used to target arretin-related molecules to therapeutically desirable sites in vivo. Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, oral, intranasal routes, and infusion into ventricles or a site of tumor removal.

The present invention is directed to genes and their encoded proteins which regulate neurite growth and the diagnostic and therapeutic uses of such proteins. The proteins of the present invention (arretin and its receptors) include proteins associated with central nervous system myelin with highly nonpermissive substrate properties, termed herein neurite growth inhibitory factors.

The present invention is also directed to antibodies to and peptide fragments and derivatives of the neurite growth inhibitory proteins and their therapeutic and diagnostic uses. These antibodies or peptides can be used in the treatment of nerve damage resulting from, e.g., trauma (e.g., spinal cord injuries), stroke, degenerative

disorders of the central nervous system, etc. In particular, antibodies to arretin proteins may be used to promote regeneration of nerve fibers. In a specific embodiment of the invention, monoclonal antibodies directed to arretin and/or its receptors may be used to promote the regeneration of nerve fibers over long distances following spinal cord damage.

The present invention is described in further detail in the following non-limiting examples. It is to be understood that the examples described below are not meant to limit the scope of the present invention. It is expected that numerous variants will be obvious to the person skilled in the art to which the present invention pertains, without any departure from the spirit of the present invention. The appended claims, properly construed, form the only limitation upon the scope of the present invention.

EXAMPLES

Example I: Isolation and characterization of a novel neurite growth inhibitory molecule from mammalian central nervous system myelin

Animals.

ICR mice and Wistar rat embryos were obtained from the animal facilities at Charles River.

Materials.

The following lectins were purchased from Sigma: Maclura pomifera (osage orange), Arachis hypogaea (PNA), Ulex europaeus (gorse), Phaseolus vulgaris PHA-L (red kidney bean), Triticum vulgaris (wheat germ), and Concanavalin A (jack bean). Laminin from EHS sarcoma, Poly-L-ornithine (PORN), Poly-L-lysine (PLL), Chondroitinase ABC (chondroitin ABC lyase, E.C. 4.2.2.4. from Proteus vulgaris,

protease-free), heparinase and PNA agrose beads were also purchased from Sigma. Horseradish peroxidase (HRP)- conjugated secondary antibodies to rabbit, rat or mouse IgG and IgM were purchased from Amersham and Jackson Labs.

Antibodies.

Monoclonal antibody 473-HD is a mouse IgM against a chondroitin sulphate epitope on mouse brain proteoglycans (Faissner *et al.*, J. Cell Biol., 126, 783-799, 1994). Rabbit polyclonal anti-versican antibodies were generated against recombinantly expressed human versican fusion proteins. We used monoclonal anti-L2 antibody (412) from rat (Kruse *et al.*, Nature, 316, 146-148, 1985) and polyclonal antibody 3F8 against phosphacan (Engel *et al.*, J. Comp. Neurol., 366, 34-43, 1996; Meyer-Puttlitz *et al.*, J. Comp. Neurol., 366, 44-54, 1996).

Multiple neurite growth inhibitory activities are present in extracts of CNS myelin after DEAE chromatography. We have previously shown that two peaks of neurite growth inhibitory activity are present in fractions of myelin extracts following DEAE chromatography (McKerracher *et al.*, Neuron, 13, 805-811, 1994). The largest of these peaks is associated with the earlier fractions eluted off the DEAE column by a 0.2 to 2 M gradient. A substantial proportion of the inhibitory activity in this peak is associated with myelin-associated glycoprotein (MAG). The inhibitory activity in column fractions was assayed by an in vitro bioassay using a neuronal cell line (NG108-15). These results suggest that molecule(s) other than MAG also contribute to the inhibitory activity associated with CNS myelin (Fig. 1).

Identification of a 70k Da protein associated with CNS myelin

In addition to MAG and the NI35/250 inhibitory molecules associated with myelin (McKerracher,

et al., 1994; Mukhopadhyay *et al.*, Neuron, 13, 757-767, 1994; Schwab *et al.*, Ann. Rev. Neurosci., 16, 565-595, 1993), three extracellular matrix, molecules namely, tenascin-C (TN-C), tenascin-R (TN-R) and chondroitin sulfate proteoglycans (CSPGs)

that are distributed in many CNS and non-CNS tissues are also known to have neurite growth inhibitory activity (Schachner *et al.,* 1994). We therefore investigated which of these inhibitory molecules are found in the two inhibitory peaks obtained after DEAE chromatography of CNS myelin extracts. DEAE column chromatographic fractions that contained the first (fractions 10) and second (fraction 26) inhibitory peaks were subjected to SDS-PAGE on a 6-16% polyacrylamide gradient gel under reducing conditions. These gels were either silver stained (Fig. 2A) or Western blotted with anti-MAG, TN-C, TN-R, and a monoclonal antibody against chondroitin sulfate (mAb 473) (Fig. 2B-E). The silver stained gels (2A) showed any bands. Anti-MAG antibody recognizes a 100 kDa band that is highly enriched in fraction10 but is much weaker in fractions 26 and 32 (Fig 2B). The intensity of the 200 and 220 kDa bands labelled with anti-TN-C was similar to that of the MAG antibody, i.e., enriched in fraction 10 (Fig. 2C). However, the 160 and 180 kDa bands recognized by the anti-TN-R antibody were present only in the total myelin extract and in fraction 10 (Fig. 2D). Interestingly, the anti-CS mAb 473 recognized 70 kDa band and a slightly small minor band in fractions 26 and 32 but not in the octylglucoside extract of myelin and/or in fraction 10. This shows that these components can only be detected immunochemically after substantial enrichment during the purification steps. These experiments show that MAG, TN-C and TN-R may contribute to the inhibitory effects of the first peak, and that MAG, TN-C and the 70 kDa bands may contribute to the second inhibitory peak. Western blots of samples of brain membranes probed with mab 412 that recognizes the HNK-1 epitope indicates that this carbohydrate epitope is not found in the 70 kDa components (data not shown).

Enzymatic hydrolysis with chondroitinase ABC and heparinase

Proteins were treated with chondroitinase ABC (0.02 U/ml) in 50 mM Tris-acetate (pH 8.0) for 2.5 h at 37°c in the presence of protease inhibitors (5 mM benzamidine, 1 mM iodoacetamide and 5 mM p-tosyl-L-lysine chloromethyl ketone, sodium salt). Heparinase digestion was done according to the manufacturer's instructions.

Purification of Arretin.

Preparation of myelin extracts and their fractionation by DEAE chromatography have been described (McKerracher *et al.,* 1994; see Fig.1). For further purification by lectin affinity chromatography, PNA-conjugated agarose beads (1.2 ml) were used. DEAE chromatographic fractions number 20 to 34 (2 ml each) were pooled (about 30 ml), diluted with 3 volume of H_2O, and loaded on the PNA-agarose column. The flow-through was reloaded three times, and the column was subsequently washed with 12 ml Hepes buffer (pH7.5, 0.08% Sodium azide, 10 mM Hepes, 0.15 mM NaCl, 0.1 mM Ca^{2+}, and 0.01 mM Mn^{2+}), followed by 12 ml of a high salt buffer (pH7.5, 2 M NaCl, and 20 mM Triethanolamine). The column was eluted with 20 ml of elution buffer (2 M NaCl, 20 mM Trithanolamine, pH7.5, and 0.5 M D-galactose). Appropriately pooled fractions were dialysed against 1000 ml of H_2O at 40°C, lyophilised, and dissolved in 1 ml of H_2O, such that the final concentration was about 0.16 M NaCl, 1.6 mM Trithanolamine, pH7.5, and 0.04 M D-galactose. Samples were aliquoted, and stored at -70°c. The protein profile was determined by SDS-PAGE on gradient gels (6 to 16% polyacrylamide) (Laemmli, U.K., Nature, 277, 680-685, 1970), by two-dimensional electrophorens and by Western blots (Towbin *et al.,* Proc. Nat, Acad. Sci. USA., 76, 4350-4354, 1979). Protein concentrations were estimated according to Bradford (1976).

Reactivity of Arretin with lectins.

Proteins tranferred to membranes were blocked with 2% bovine serum albumin (BAS) in TBS buffer (20 mM Tris-HCl, 500 mM NaCl, pH7.5) for 1 h, and incubated separately with 6g/ml of different HRP- or biotin-conjugated lectins for 2 h. The membranes were washed with TTBS (20 Mm Tris-HCl, 500 mM NaCl, 0.05% Tween-20, pH7.5) for 1h and complexes were detected by ECL (DU PONT) or the AP-ABC (VECTOR) Kit according to the manufacturer's instructions. As positive controls for lectin binding, several sugars, including galactose, glucose, glucosamine, galactosamine, fucose, and mannose (at 20 mg/ml), were applied as spots on nitrocellulose.

Purification by Lectin affinity chromatography

To further purify the 70 kDa components from DEAE fractions containing the second inhibitory peak, we screened the ability of the components to bind the following lectins: Maclura pomifera (osage orange), Arachis hypogaea (PNA), Ulex europaeus uea I (gorse or furze). Phaseolus vulgaris (PHA-L), Triticum vulgaris (wheatgerm agglutinin) and Concanavalin A (Con-A). Nitrocellulose membranes electro blotted with pooled DEAE fractions 20 to26 after protein separation by SDS-PAGE were probed with the various lectins. All the lectins except Con-A bound only to the 70 kDa bands (not shown).

We next tested whether the 70 kDa components could be purified by binding to lectin. For this, PNA-conjugated agarose beads were chosen. Fractions 20 to 26 obtained from DEAE column chromatography of bovine CNS myelin extracts were pooled and incubated with PNA-conjugated beads in an Eppendorf tube. After washing the beads, the proteins bound to the PNA-beads were separated by SDS-PAGE, electrophoretically blotted onto nitrocellulose membrane and probed with anti-MAG, TN-C and the 473 antibodies. As expected only the 70 kDa bands were recognized by the mAb 473. No labeling was observed with the other two antibodies, indicating that PNA lectin can be used to separate the 80 kDa molecule from MAG and TN-C (not shown).

A two-step purification of the 70 kDa components was therefore attempted. Octylglucoside extracts of bovine CNS myelin were passed though a DEAE column, and the material eluted by a NaCl gradient, and fractions 20-34 were pooled. The pooled fractions were then subjected to PNA-affinity chromatography. The material eluted from the PNA column was separated on a SDS-PAGE gradient gel (6-16%

acrylamide) under reducing conditions. The gels were then stained with silver, or Western blotted and probed with anti-MAG, TN-C and 473 antibodies. A 70 kDa doublet was seen after Amido black staining (Fig. 5A). This major band was recognized only by the 473 anti-CS antibody (Fig. 3A), but not by anti-MAG (Fig. 3B) or anti-TN-C antibodies (not shown). The minor component just below the major band was not visible in this preparation.

The 70 kDa components are novel phosphocan-versican-related molecules. We further investigated whether the 70 kDa bands purified from CNS myelin shared epitopes with other known CSPGs. On Western blots of the DEAE chromatographic fractions the 70 kDa bands also reacted with polyclonal antibodies against phosphacan and recombinant versican (Fig. 4A and B). Both these antibodies plus the 473 anti-CS recognized the 70 kDa PNA affinity purified polypeptides (Fig. 4C, D, E). After chondroitinase ABC treatment, the major 70 kDa proteins were found to have an apparent Mr of 50 kDa (Fig. 5A) which did not react with the anti-CS mAb 473 (not shown), but did react with anti-phosphacan (Fig. 5B) and anti-versican. Since native phosphacan has a molecular weight of 500-600 kDa (core protein 400 kDa), and versican is a very large proteoglycan with a molecular weight of 900 kDa (core protein 400 kDa), the 70 kDa components that we have isolated from CNS myelin are likely to be novel proteins. We call these proteins arretin (collectively). The 2 bands may represent 2 isoforms, or the smaller component may be an altered version of the larger, due to degradation.

The 70 kDa proteins inhibit neurite growth. The present invention involved a test that examined effects of the 70 kDa myelin-derived proteins in modulating neurite growth from rat hippocampal and cerebellar granule celleurons. The 70 kDa proteins inhibited neurite growth from neonatal rat cerebellar and hippocampal neurons (Figs. 7 and 8), as well as from cultured NG108-15 cells (Fig. 9). This inhibitory activity was lost after heat denaturation. These result indicate that novel myelin-associated 70 kDa proteins are inhibitors of neurite growth, and are likely to be largely responsible for the activity associated with the second inhibitory peak in fractions obtained after DEAE separation

of CNS myelin extracts. The present invention comprises these new inhibitors collectively termed as arretin.

Assays for repulsion of growth cones and cell bodies.

Tissue culture dishes (Becton Dickinson) with 24 wells were coated with methanol-solubilized nitrocellulose according to Lagenaur and Lemmon (1987) and air-dried in a sterile hood. For assays addressing the effect of arretin on growth cones, nitrocellulose and poly-L-lysine (PLL 0.01%) coated dishes were used as described (Xiao *et al.*, Neurosci., 8, 766-782, 1996). The dishes were washed three times with PBS and dried in a sterile hood. Different test proteins (arretin, denatured (80°c for 30 min) arretin, TN-R, and laminin), each at concentrations of 2 nM, 10nM, and 50nM, were applied in duplicate as 2.5 µl single spots to the dishes and incubated overnight at 37°c in a humidified atmosphere.

Determination of substrate coating efficiency was been described by Xiao et al., 1996. Before plating the NG108 cells or cerebellar neurons, the dishes were washed with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (CMF-HBSS). Explants were prepared from cerebella of 6 to 7-day-old mice and maintained in a chemically defined medium (Fischer *et al.,* J. Neurosci., 6, 605-612, 1986; Fischer, G., Neurosci. Lett., 28, 325-329, 1982). Explants were allowed to grow neurites for 72 h and then fixed with glutaraldehyde in PBS at a final concentration of 2.5%.

After fixation, cultures were stained with 0.5% toluidine blue (Sigma) in 2.5% sodium carbonate, washed five times with water and air dried. All experiments were performed at least three times. Assay for neurite outgrowth. Hippocampal neurons from 18- to 19-day-old rat embryos were prepared as described (Keilhauer *et al.*, Nature, 316, 728-730, 1985; Lochter *et al.*, J. Cell Biol., 113, 1159-1171, 1991; Dorries *et al.*, 1995 ?). For the assays on neurite outgrowth, hippocampal neurons were maintained in chemically defined medium (Rousselet *et al.*, Ann. Rev. Cell Biol., 129, 495-504, 1988; Lochter and Schachner, J. Neurosci., 13, 3986-4000, 1993; Xiao *et al.*, 1996).

Briefly, 96-well plates (Nunc) were pretreated with 5g/ml poly-L-ornithine (PORN) for 1 to 2 hours at 37°c, washed twice with water and air-dried. Proteins at concentrations of 2 nM, 10 nM, and 50 nM were coated on the dried surfaces overnight at 37°c in a humidified atmosphere. Determination of substrate coating efficiency as described Xiao *et al.*, 1996. The plates were washed three times with CMF-HBSS and hippocampal neurons prepared from 18- to 19-day-old rat embryos (Keilhauer *et al.*, 1985; Lochter *et al.*, 1991; Dorries *et al.*, 1995) were plated at a density of 3,000 cells per well in 100µl a chemically defined medium (Rousselet *et al.*, 1988; Lochter and Schachner, 1993; Xiao *et al.*, 1996). After 12 h, cells were fixed without a preceding washing step by gentle addition of 25% glutaraldehyde to a final concentration of 2.5%. After fixation, cultures were stained with toluidine blue and morphological parameters were quantified with an IBAS image analysis system. For morphometric analysis, only cells without contact with other cells were evaluated. Neurites were defined as those processes with a length of at least one cell body diameter. The total neurite length per cell was determined by analysing 50 cells in each of two wells. To determine the number of cells with neurites, 100 neurons in each of two wells were counted per experiment. Raw data from at least three independent experiments were analyzed by ANOVA and by the Newman-Keuls test with $P < 0.05$ and $P < 0.01$ being considered significant or highly significant, respectively. All graphs comprise data derived from at least three independent experiments.

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications to the invention to adapt it to various usages and conditions. Such changes and modifications are properly, equitably, and intended to be within the full range of equivalence of the following claims.

CLAIMS

1. A protein consisting of a molecule, derivative or fragment thereof, characterized by the following properties:
   a) said protein has an apparent molecular weight of 70 kDa; and
   b) said protein mediates inhibition of neurite outgrowth.

2. A nucleic acid sequence encoding the protein, derivative, or fragment thereof as in claim 1.

3. An isolated receptor that binds the protein of claim 1.

4. A nucleic acid sequence encoding the receptor, derivative, or fragment thereof as in claim 3.

5. An antagonist comprising an antibody or a binding fragment thereof, directed toward the protein, derivative or fragment of claim 1.

6. A fragment, analog or derivative of the protein of claim 1, which interferes with arretin mediated inhibition as competitive but non-functional mimics of endogenous arretin.

7. An antagonist comprising a peptide or its analog modelled on a sequence of the protein of claim 1 which serves as an antagonist of the arretin-receptor interaction.

8. An antagonist comprising blocking peptides or small molecules modelled on an extracellular region of the protein of claim 1 which mediates inhibitory activity.

9. An antagonist compromising a peptide, peptidiomimetic compound, or derivative thereof that is capable of neutralizing the inhibitory substrate property of the protein of claim 1 which said neutralization is detected by

observing the ability of said antagonist to suppress inhibition of neuron growth, comprising the steps of:

a) culturing neurons on a growth substrate that incorporates a growth-inhibiting amount of arretin; and

b) exposing said cultured neurons to said antagonist agent in an amount and for a period sufficient prospectively to permit growth of said neurons.

10. An antagonist compromising a peptide, peptidiomimetic compound, or derivative thereof that is capable of neutralizing the inhibitory substrate property of the protein of claim 1, said neutralization detected by observing the ability of said antagonist to suppress inhibition of neuron growth, comprising the steps of:

a) culturing neurons on a growth substrate that incorporates a growth-inhibiting amount of arretin; and

b) exposing said cultured neurons to said antagonist agent in an amount and for a period sufficient prospectively to block the growth cone collapse response by arretin.

11. An antagonist comprising a chemical compound possessing the ability to alter the biological activity of the neuronal receptor for the protein of claim 1 such that growth of neurons or their axons is suppressed.

12. An isolated and purified antibody or binding fragment thereof, capable of neutralizing the biological activity of the protein of claim 1, wherein the antibody is a monoclonal antibody.

13. A hybridoma cell line which produces the monoclonal antibody of claim 12.

14. An isolated and purified antibody or binding fragment thereof, capable of

neutralizing the biological activity of the protein of claim 1, wherein the antibody is a polyclonal antibody.

15. The use of the protein of claim 1, biologically active variants or fragments thereof, for raising antibodies or ligands thereof which overcome growth inhibition.

16. A polypeptide having an amino acid sequence or a subsequence thereof wherein the polypeptide has from about 18 to 23 amino acid residues such that antibodies having antagonistic activity to the protein of claim 1 can be raised against said polypeptide.

17. A hybridoma cell line producing an antibody that specifically binds arretin.

18. A pharmaceutical composition comprising an antibody having the property of inhibiting arretin activity wherein arretin has an apparent molecular weight of 70 kDa, wherein the antibody of arretin is isolated from the blood serum of an animal to which said arretin has been previously added.

19. An arretin antagonist formulated as a pharmaceutical composition containing one or more arretin antagonists in an amount effective to suppress arretin-mediated inhibition of nerve growth, in combination with a suitable pharmaceutical carrier, wherein said antagonist is selected from the group comprising a fragment of arretin, a peptide, or a chemical molecule.

20. A pharmaceutical composition for nerve regeneration treatment of a patient comprising an effective amount of an arretin antagonist in a suitable pharmacologic carrier.

21. A pharmaceutical composition for treatment of a patient with damage to the

central nervous system comprising an effective amount of a substance that is capable of neutralizing the inhibitory substrate property of arretin in which neutralization is detected by observing the ability of the antagonist to suppress inhibition of neuron growth, comprising the steps of:

a) culturing neurons on a growth substrate that incorporates a growth-inhibiting amount of arretin; and

b) exposing said cultured neurons to the arretin antagonist agent in an amount and for a period sufficient prospectively to permit growth of said neurons.

22. The pharmaceutical composition of claim 21, wherein the antagonist substance is an antibody or binding region thereof.

23. The pharmaceutical composition of claim 21, wherein the damage is due to infarction, traumatic injury, surgical lesion or a degenerative disorder of the central nervous system.

24. The pharmaceutical composition of claim 21, wherein the damage has occurred to the spinal cord.

25. The pharmaceutical composition of claim 21, wherein the antibody is administered by the introduction into the patient of an antibody-secreting cell.

26. A pharmaceutical composition for treatment of a patient with damage to the central nervous system or the peripheral nervous system comprising an effective amount of arretin antagonist consisting of a peptide, peptidiomimetic compound, or derivative thereof that is capable of neutralizing the inhibitory substrate property of arretin in which neutralization is detected by observing the ability of the antagonist to suppress inhibition of neuron growth, comprising the steps of:

a) culturing neurons on a growth permissive substrate that incorporates a growth-inhibiting amount of arretin; and

b) exposing said cultured neurons to the arretin antagonist agent in an amount and for a period sufficient prospectively to permit growth of said neurons.

27. A method effective to suppress the inhibition of neuron growth, comprising the step of delivering an arretin antagonist to the nerve growth environment in an amount effective to reverse said inhibition.

28. A method according to claim 27, wherein said arretin antagonist is selected from an arretin antibody or a binding fragment of said antibody, an arretin fragment, a derivative of an arretin fragment, an analog of arretin or of an arretin fragment or of said derivative, and a pharmaceutical agent, and is further characterized by the property of suppressing arretin-mediated inhibition of neurite outgrowth.

29. A method according to claim 28, wherein said arretin antagonist is an arretin antibody or a binding fragment thereof.

30. A method according to claim 27, 28, or 29, wherein said arretin antagonist is delivered to the growth environment of a CNS neuron requiring growth or regeneration as a result of spinal cord injury, spinal cord lesions, surgical nerve lesions, damage secondary to infarction, infection, exposure to toxic agents and malignancy.

31. A method according to claims 27, 28, or 29, wherein said arretin antagonist is delivered to a patient having a medical condition selected from Strokes, Alzheimer's disease, Down's syndrome, Creutzfeldt-Jacob disease, kuru, Gerstman-Straussler syndrome, scrapie, transmissible mink encephalopathy,

Huntington's disease, Riley-Day familial dysautonomia, multiple system atrophy, amyotropic lateral sclerosis or Lou Gehrig's disease, progressive supranuclear palsy, Parkinson's disease.

32. The use of the antagonist of claim 18 to treat a patient with damage to the central nervous system comprising administering to the patient an effective amount of monoclonal antibody directed towards arretin, wherein arretin has an apparent molecular weight of 70 kDa, and said antibody blocks the inhibitory effects of arretin, or a fragment thereof containing the binding region.

33. An assay method useful to identify arretin antagonist agents that suppress inhibition of neuron growth, comprising the steps of:

a) culturing neurons on a growth substrate that incorporates a growth-inhibiting amount of arretin; and

b) exposing the cultured neurons of step a) to a candidate arretin antagonist agent in an amount and for a period sufficient prospectively to permit growth of said neurons;

thereby identifying as arretin antagonists said candidates of step b) which elicit neurite outgrowth from said cultured neurons of step a)

34. An assay method as in claim 33, wherein the cultured neurons are selected from the group comprising primary neurons or neuronal cell lines.

35. A method for screening for compounds that stimulate cell adhesion and neurite growth, comprising the steps of:

e) coating a growth permissive substrate with a growth-inhibiting amount of arretin;

f) adding a test compound and neuronal cells to the arretin-coated substrate;

g) washing to remove unattached cells; and

h) measuring the viable cells attached to the substrate,

thereby identifying the cell adhesion candidates of step b) which elicit neurite outgrowth from the cultured neurons of step a).

36. An assay method useful to identify arretin antagonist agents that suppress inhibition of neuron growth, comprising the steps of:

a) culturing cells that extend cytoplasmic processes whose growth is inhibited by arretin, on a growth substrate that incorporates a growth-inhibiting amount of arretin; and

b) exposing the cells to a candidate arretin antagonist agent in an amount and for a period sufficient prospectively to permit growth of said cells;

thereby identifying as arretin antagonists said candidates of step b) which elicit changes in cell attachment, cell spreading, cell migration, cell invasiveness or cell morphology from said cultured cells of step a)

37. A method for screening for compounds that stimulate neurite growth, comprising the steps of:

a) coating a growth permissive substrate with a growth-inhibiting amount of arretin; and

b) adding a test compound and arretin-growth-sensitive cells to the arretin-coated substrate;

c) washing to remove unattached cells;

d) measuring the viable cells attached to the substrate,

thereby identifying the cell adhesion candidates of step b) which elicit changes in cell attachment within the cultured cells of step a).

38. A method for inhibiting neuron growth, comprising the step of introducing into the neuron growth environment a growth-inhibiting amount of a neuron growth inhibitor selected from arretin and an arretin agonist.

39. A method according to claim 38, wherein said inhibitor is arretin.

40. A method according to claim 39, wherein said inhibitor is an arretin agonist having arretin-biological activity of inhibiting neurite outgrowth from neurons cultured on a permissive substrate, and is selected from an arretin fragment, an analog of arretin or of the arretin fragment, a derivative of either arretin, the arretin fragment or said analog, an anti-idiotypic arretin antibody or a binding fragment thereof, and a pharmaceutical agent.

41. A method according to claim 40, wherein said arretin agonist is the arretin ectodomain.

42. A method according to claims 38, 39, 40 or 41, wherein said inhibitor is delivered to a patient afflicted with a medical condition selected from epilepsy, neuroblastoma and neuromas.

43. An antisense oligonucleotide having a sequence capable of binding specifically with any sequences of an mRNA molecule which encodes arretin so as to prevent translation of the mRNA molecule.

44. An antisense oligonucleotide having a sequence of binding specifically with any sequences of a cDNA molecule coding for arretin.

45. An antisense oligonucleotide of claim 43 comprising chemical analogues of nucleotides.

46. A pharmaceutical composition comprising an amount of the oligonucleotide of claim 43 effective to reduce expression of arretin by passing through a cell membrane and binding specifically with mRNA encoding arretin in the cell so as to prevent its translation and a pharmaceutically acceptable hydrophobic

carrier capable of passing through a cell membrane.

47. A pharmaceutical composition of claim 46, wherein the oligonucleotide is coupled to a substance which inactivates mRNA.

Figure 1A

A

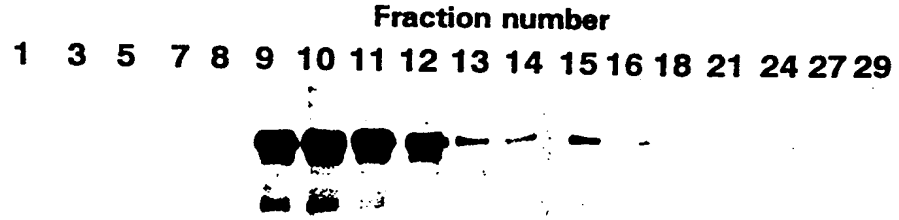
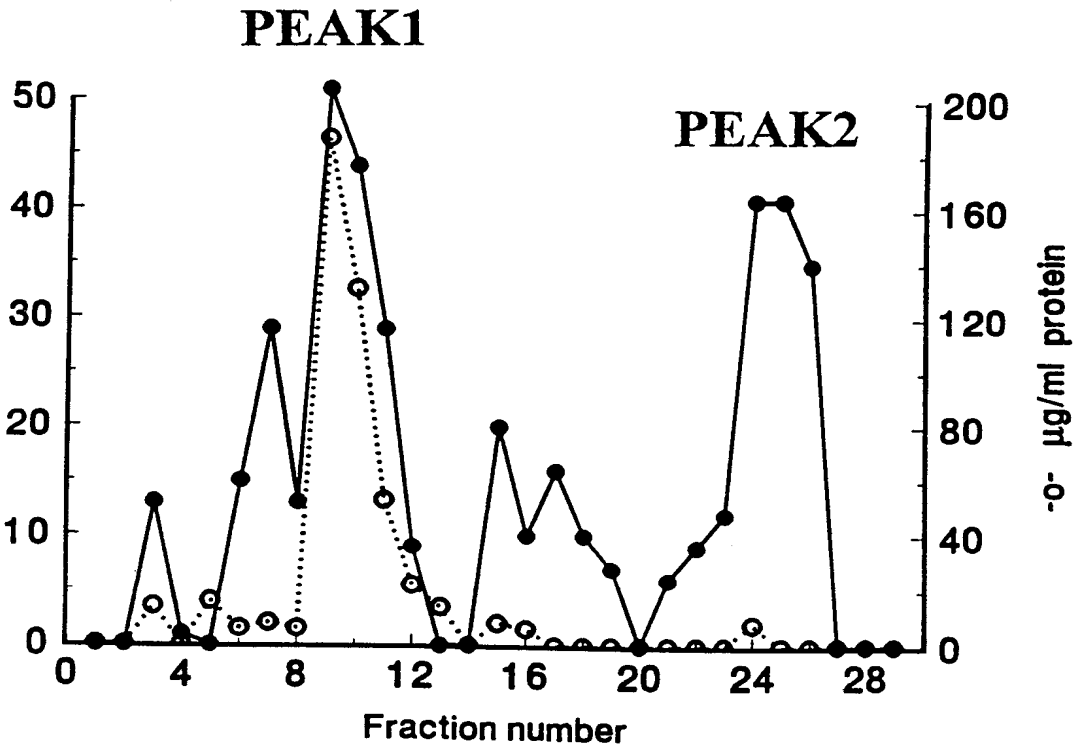


Figure 1B

B



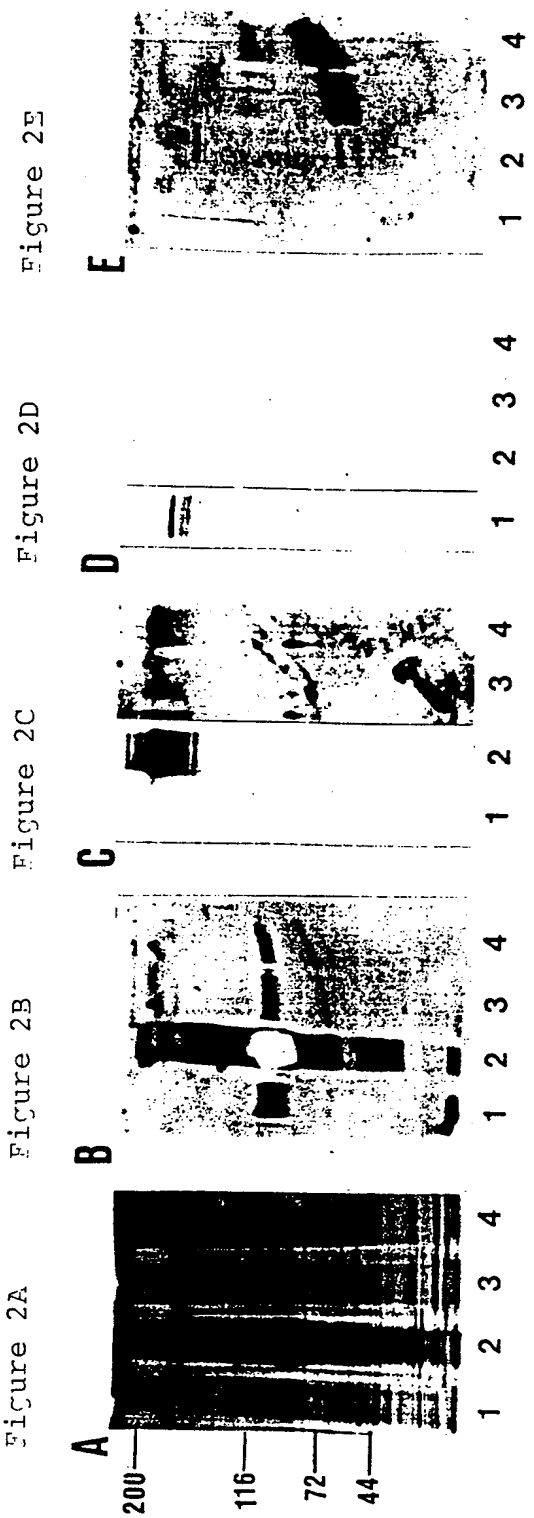


Figure 2A Figure 2B Figure 2C Figure 2D Figure 2E

Figure 3A

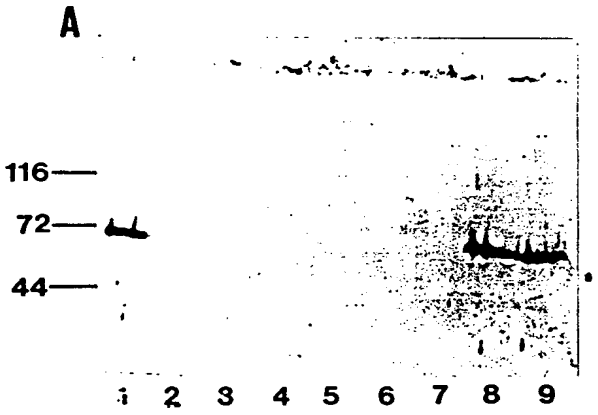


Figure 3B

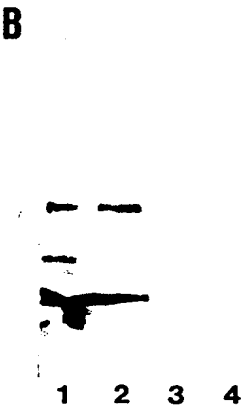
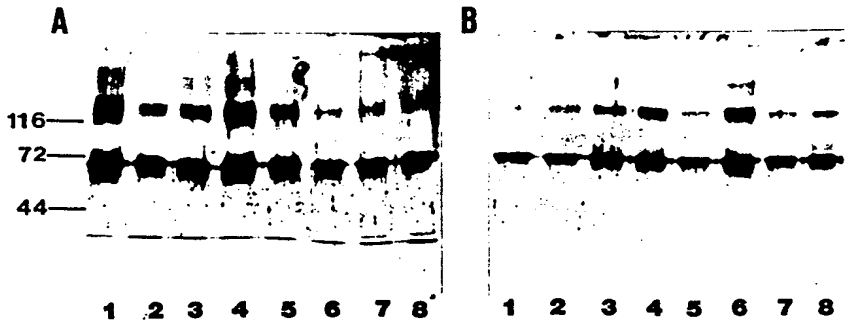


Figure 4A Figure 4B



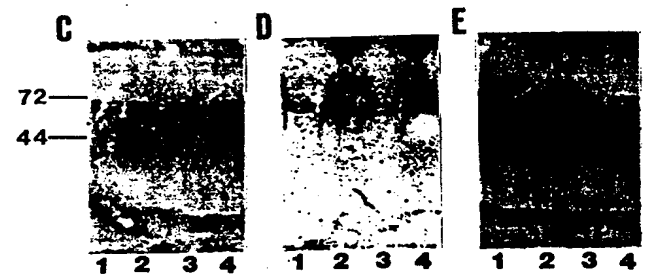


Figure 4C Figure 4D Figure 4E

Figure 5A Figure 5B

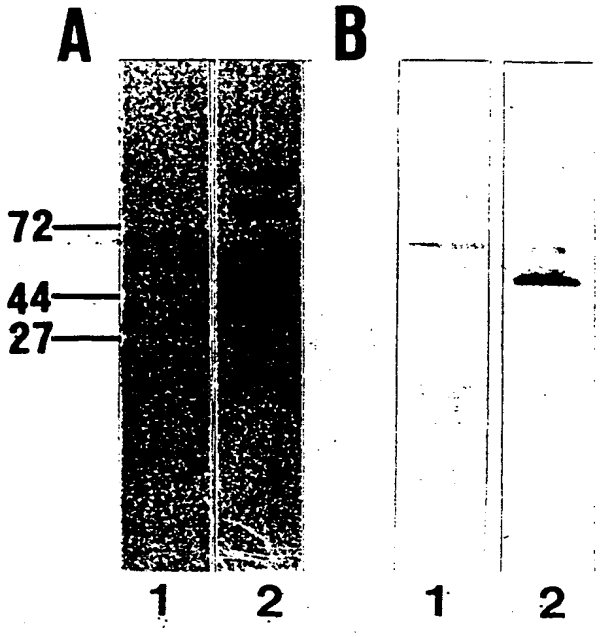


Figure 6

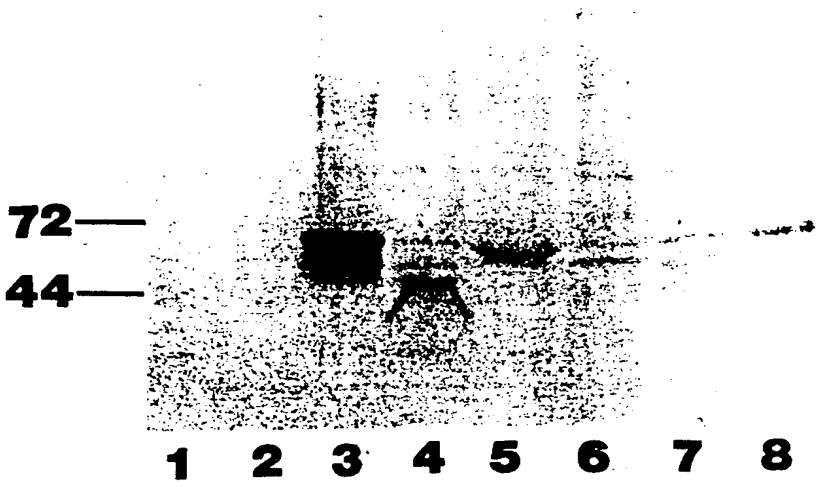


Figure 7

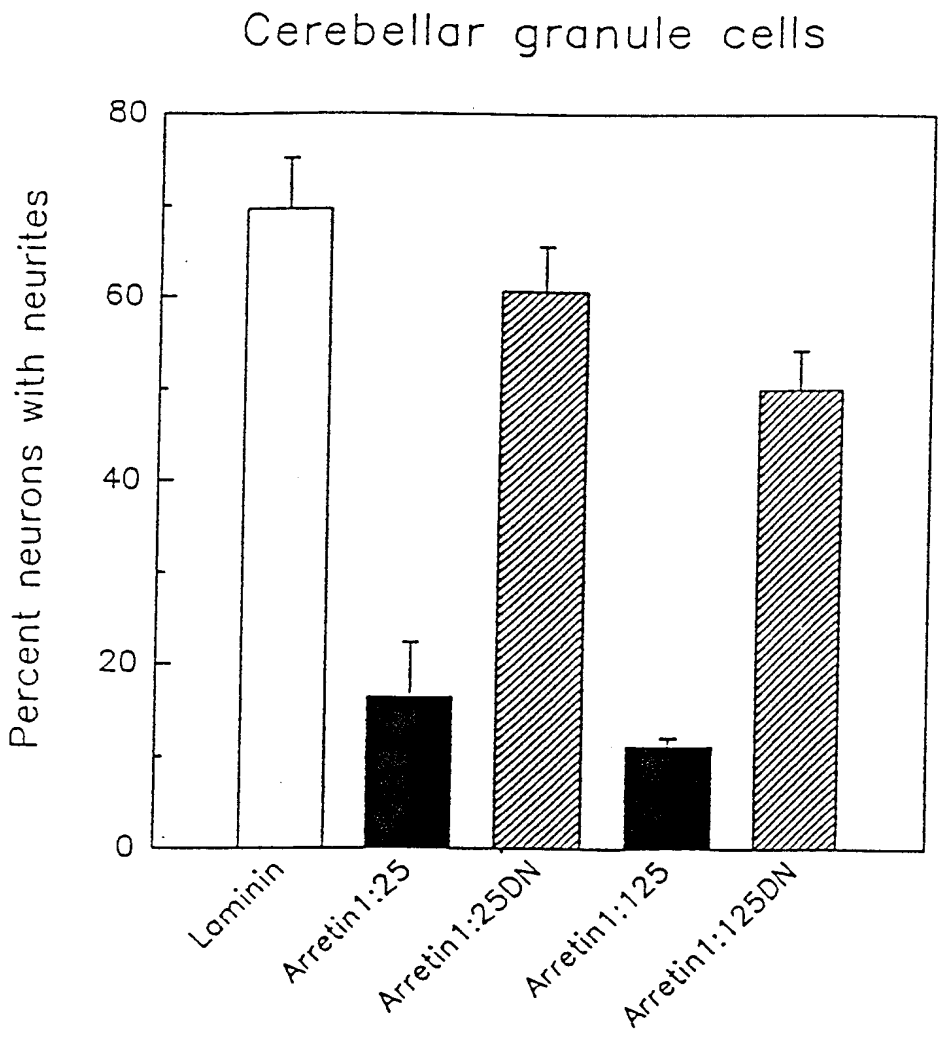

Cerebellar granule cells
Percent neurons with neurites
80
60
40
20
0
Laminin
Arretin1:25
Arretin1:25DN
Arretin1:125
Arretin1:125DN

Figure 8

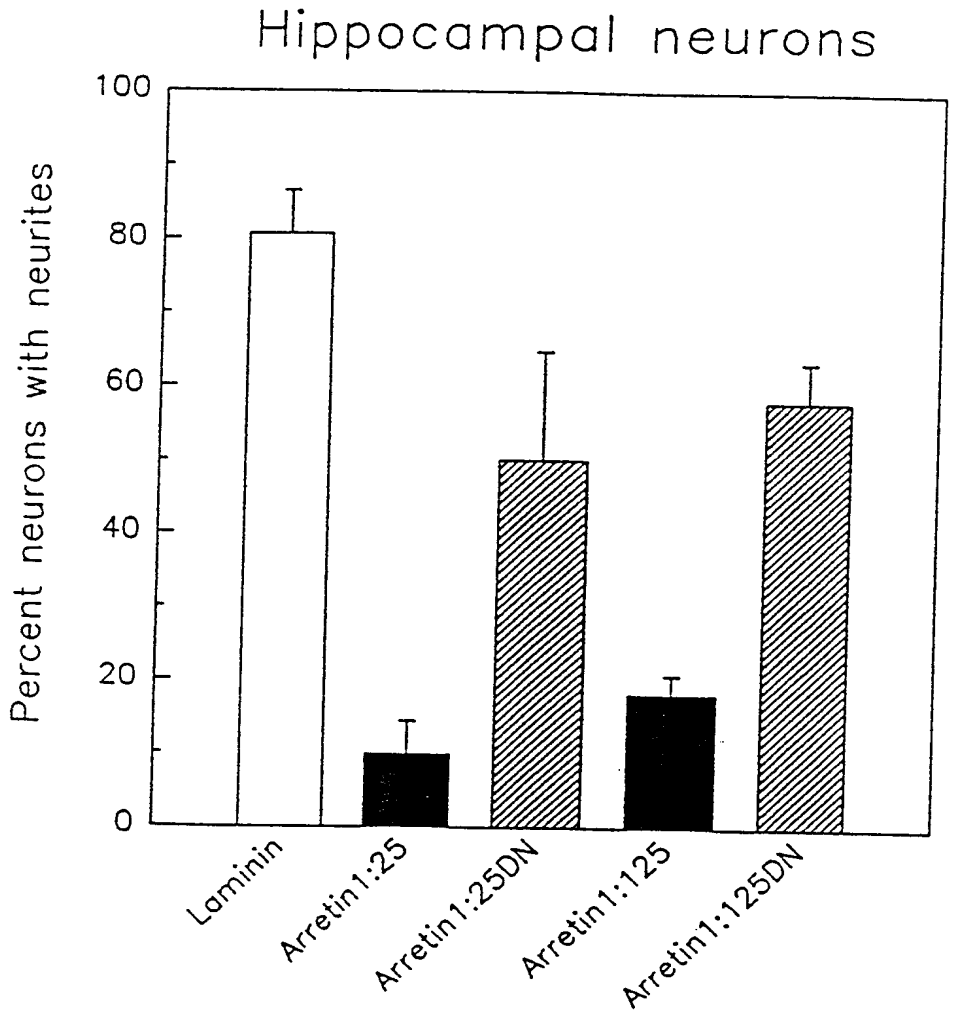

Hippocampal neurons
Percent neurons with neurites
100
80
60
40
20
0
Laminin
Arretin1:25
Arretin1:25DN
Arretin1:125
Arretin1:125DN

Figure 9

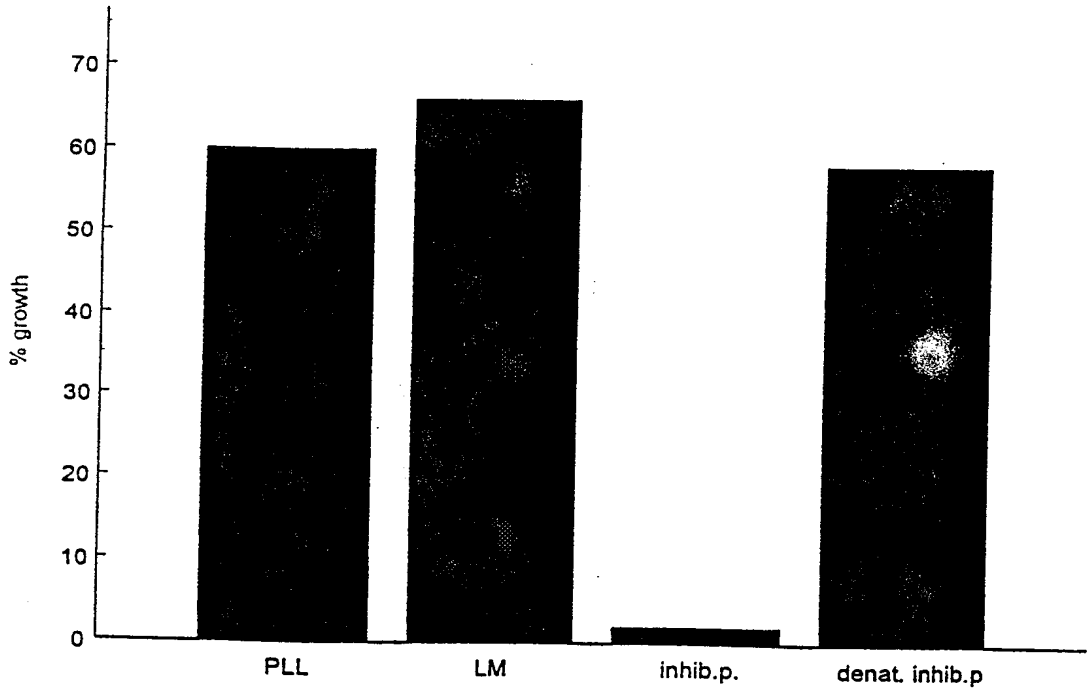


Figure 10

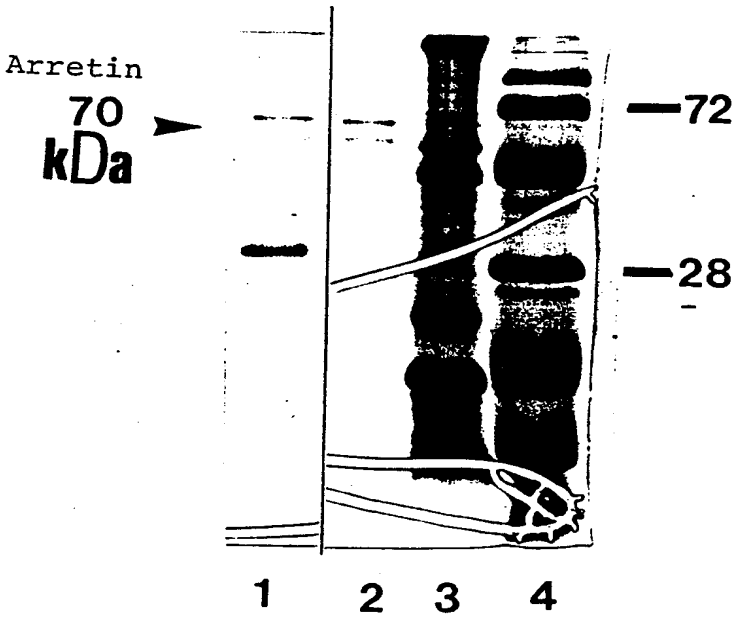


Figure 11

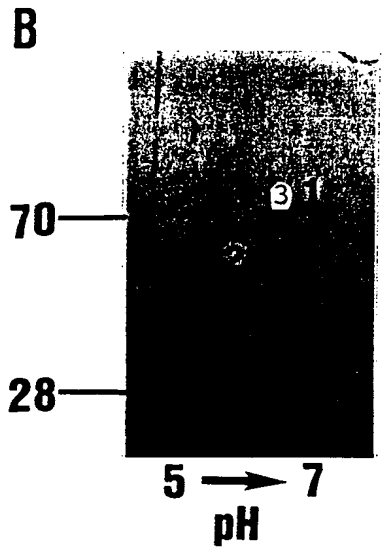


Figure 12A

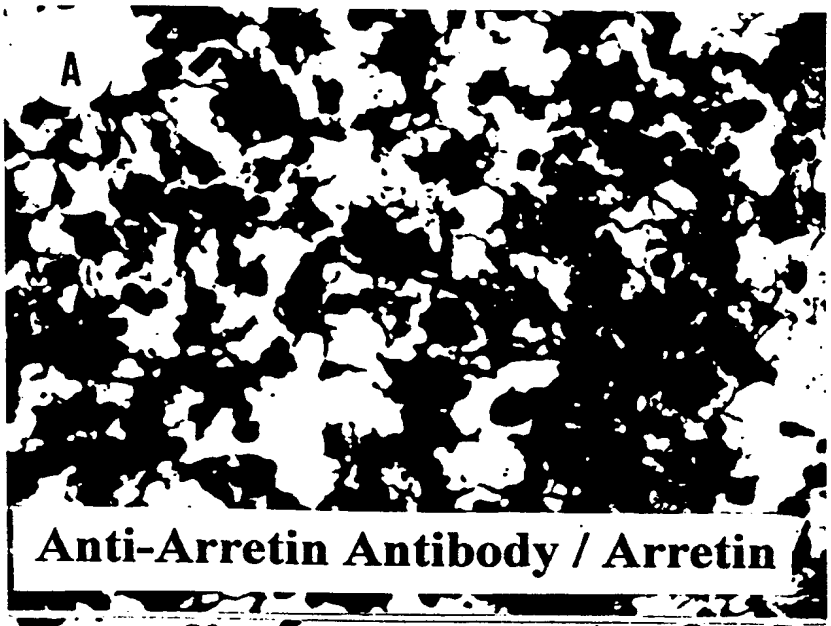


Figure 12B

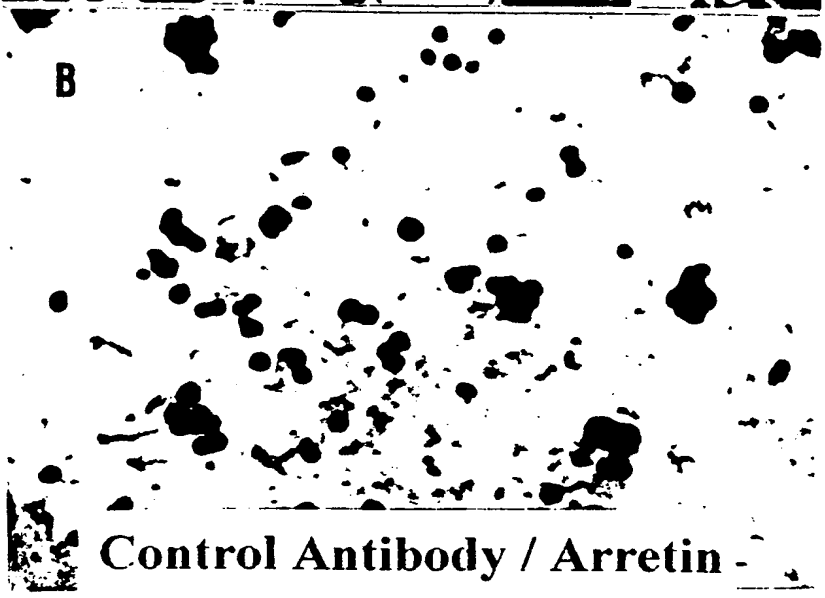


Figure 13

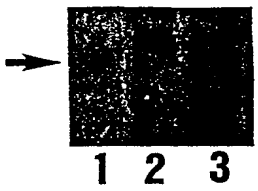
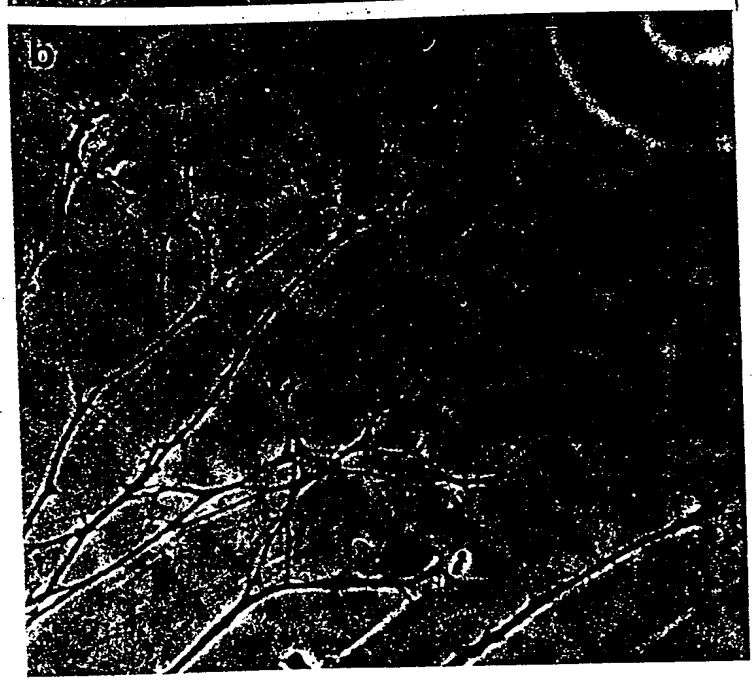


Figure 14A

Figure 14B



INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/CA 97/00868

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/11 C07K14/47 C07K16/22 A61K31/70 A61K39/395
A61K38/17 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 95 22344 A (UNIV MCGILL ;MCKERRACHER LISA JOAN (CA); DAVID SAMUEL (CA); BRAUN) 24 August 1995 see the whole document ---	1-47
A	FRIEDLANDER ET AL.: "THE NEURONAL CHONDROITIN SULFATE PROTEOGLYCAN NEUROCAN BINDS TO THE NEURAL CELL ADHESION MOLECULES Ng-CAM/L1/NILE AND N-CAM, AND INHIBITS NEURONAL ADHESION AND NEURITE OUTGROWTH" THE JOURNAL OF CELL BIOLOGY, vol. 125, no. 3, 1994, pages 669-680, XP002062504 see page 670 left-hand column, paragraph 1 and see page 677, left-hand column, paragraph 1 --- -/--	1-47

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search
20 April 1998

Date of mailing of the international search report
20. 05. 1998

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Hagenmaier, S

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INTERNATIONAL SEARCH REPORT

In ıtional Application No
PCT/CA 97/00868

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BODE-LESNIEWSKA ET AL.: "Distribution of the large aggregating proteoglycan versican in adult human tissues" THE JOURNAL OF HISTOCHEMISTRY AND CYTOCHEMISTRY, vol. 44, no. 4, - April 1996 pages 303-312, XP002062505 cited in the application see the whole document	1-47
A	WO 95 20397 A (UNIV NEW YORK) 3 August 1995 see the whole document	1-47
A	WO 94 03601 A (UNIV NEW YORK) 17 February 1994 see the whole document	1-47
A	WO 96 32959 A (ACORDA THERAPEUTICS) 24 October 1996 see the whole document	1-47
A	WO 96 32476 A (MOUNT SINAI HOSPITAL CORP ;ROACH ARTHUR (CA); LOZANO ANDRES (CA);) 17 October 1996 see the whole document	1-47
A	WO 95 26201 A (JOLLA CANCER RES FOUND) 5 October 1995 see the whole document	1-47
A	WO 94 17831 A (ZUERICH ERZIEHUNGSDIREKTION) 18 August 1994 see the whole document	1-47
A	SCHWAB M E ET AL: "INHIBITORS OF NEURITE GROWTH" ANNUAL REVIEW OF NEUROSCIENCE, vol. 16, 1 January 1993, pages 565-595, XP000576555 cited in the application see the whole document	1-47
A	ESKO J.: "GENETIC ANALYSIS OF PROTEOGLYCAN STRUCTURE, FUNCTION AND METABOLISM" CURRENT OPINION IN CELL BIOLOGY, vol. 3, 1991, pages 805-816, XP002062506 see the whole document	1-47

2

INTERNATIONAL SEARCH REPORT

In ational application No.
PCT/CA 97/00868

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 27-32,42(completely), 38-41 (partially, as far as an in vivo method is concerned) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. ional Application No
PCT/CA 97/00868

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9522344 A	24-08-95	AU 1703595 A	04-09-95
		CA 2183630 A	24-08-95
WO 9520397 A	03-08-95	US 5625040 A	29-04-97
		AU 1693995 A	15-08-95
WO 9403601 A	17-02-94	AU 4801393 A	03-03-94
		CA 2141600 A	17-02-94
		US 5648465 A	15-07-97
WO 9632959 A	24-10-96	AU 5557296 A	07-11-96
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WO 9632476 A	17-10-96	AU 5264496 A	30-10-96
		CA 2174025 A	14-10-96
		CA 2217731 A	17-10-97
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		EP 0634939 A	25-01-95
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		ZA 9400887 A	23-08-94